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ACTION POTENTIALS FROM SOME INVERTEBRATE NON-STRIATED MUSCLES

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THIRTEEN FIGURES

Propagation in non-striated muscle has been variously attributed to conduction in muscle fibers, conduction in nerve elements only, conduction from muscle fiber to muscle fiber by protoplasmic connections or by diffusion of a chemical agent. Action potentials from non-striated muscles are usually complex, having both fast and slow components, and records have not been obtained from single fibers. The non-striated muscles of some invertebrates are advantageous for study of propagation because the fibers are relatively long and some of them occur in long, thin bundles. The following muscles were used: proboscis retractors of *Phascolosoma gouldi,* lantern retractors and longitudinal body wall muscles of *Thyone briareus* and byssus retractors of *Mytilus edulis.*

Fast (spike) and slow potential waves were described by DuBuy ('36) from *Thyone* lantern retractors; these were conducted in a system which was blocked by curare. Impulses in the byssus retractor of *Mytilus* are single, are propagated at 13 to 22 cm/sec.; the potentials summate and fuse on repetitive stimulation (Fletcher, '36). Simple action potentials were recorded from the proboscis retractors of *Sipunculus* by Fuchs ('10).

MATERIALS AND METHODS

Action potentials were recorded by means of a direct-coupled amplifier and contractions by means of a vacuum

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tube transducer, both amplifier and transducer feeding directly to a cathode ray oscilloscope. Muscles were usually tied at both ends and submerged in light mineral oil; the temperature was 22–25°C. Stimulating and recording electrodes were chlorided silver making connection with the muscle through sea-water bridges by small wicks which were moved by means of micromanipulators. Stimuli were square pulses from a multivibrator and they were made diphasic by an isolation transformer, except when strength-duration curves were being obtained.

Action potentials and contractions in proboscis retractors of Phascolosoma

The sipunculoid worm Phascolosoma has 4 proboscis retractors which have their insertion at the junction between proboscis and esophagus and their origin on the body wall. They are innervated from the cerebral ganglion imbedded near the insertion of the muscle and this ganglion receives fibers from the ventral nerve cord by two nerves lying on the surface of the esophagus. Except when the nerve or ganglion was stimulated, the muscle was tied below the ganglion.

I. Stimulation of the muscle. (a) Threshold and general character of response. The threshold for direct electrical stimulation of the muscle was as much as 10 to 20 times less than for stimulation by way of the nerve or ganglion, as had been noted by Uexküll (1896). The muscle remained excitable and reproducible responses were obtained for many hours while the excitability of the nerve or ganglion rapidly diminished. The threshold was similar and the responses virtually the same irrespective of which end of the muscle was stimulated. Muscles remained excitable during 3 to 5 days in the refrigerator after isolation. It cannot be said with certainty whether nerve elements in the muscle were being stimulated but it seems more likely that the muscle fibers were stimulated directly.

Two types of action potential were recorded, a fast spike and a slow wave of negativity. The slow wave facilitated greatly and its threshold was difficult to determine; however it sometimes was the same, sometimes lower (fig. 1), but usu-

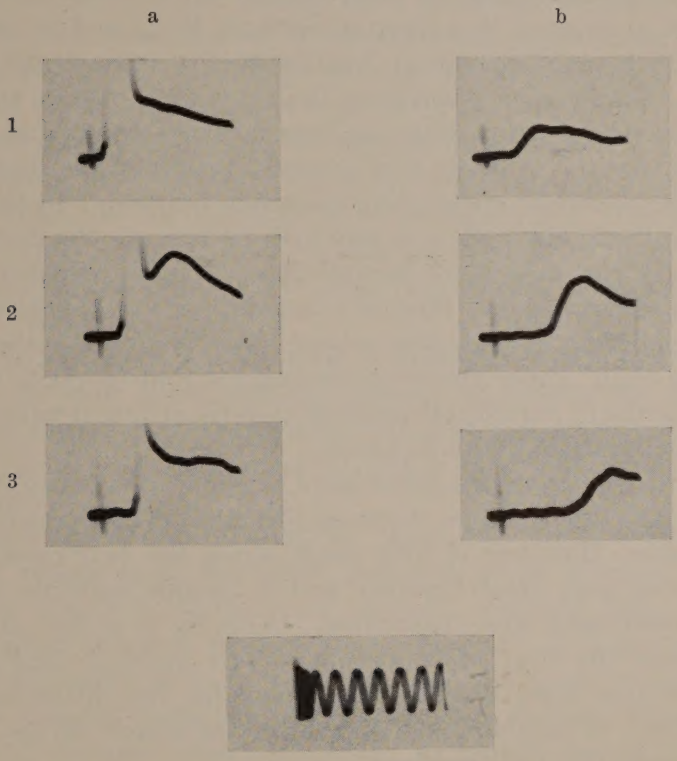

Fig. 1 Propagation of action potentials in Phascolosoma muscle. Series (a) at intensity eliciting both fast and slow waves, series (b) at lower intensity eliciting slow wave only. Conduction distance in (1) 4 mm, (2) 11 mm, (3) 20.5 mm. Time record 60/sec.

ally higher than the threshold of the spike. Separation on the basis of threshold was usually such that the spike showed little or no gradation with stimulus intensity, that is, it appeared to be nearly all-or-none. However the slow wave graded very much according to the stimulus, either duration, intensity or frequency. Movement of the active recording

electrode along the muscle failed to reveal any evidence of focal points at which either type of potential might originate.

(b) Shape of action potentials. The shape of both spike and slow wave varied according to electrode position, as illustrated by recording between the end of the muscle (either crushed or injured by a drop of isotonic KCl) and an active lead which could be moved relative to the stimulating electrodes. Under such conditions the slow wave was a simple monophasic deflection with rise time of 0.02 seconds and time for half decline of about 0.04 seconds, provided the distance from the proximal stimulating electrode (cathode) to the active recording electrode was less than 10 mm. As the distance increased, the slow wave became irregularly broadened and showed several peaks. Similar observations were made when the stimulating cathode was moved away from the active recording lead. These observations suggest that the slow wave is conducted in parallel elements of slightly different velocities which separate out with distance.

The fast wave, on the other hand, was not affected in shape by varying the distance between stimulating and recording electrodes. However it was single when the two recording electrodes were close together and it became multiple when these electrodes were separated. Often in a series at low frequency the first spike response was multiple and subsequent ones were single. The reason for the difference in the effects of conduction distance on the shape of the fast and slow waves is not evident.

Both spike and slow wave showed varying degrees of diphasicity according to the state of the "indifferent" end of the muscle. Invariably, however, if the spike was diphasic, so also was the slow wave. The slow wave has a distinct rising phase and can be obtained independently of the spike; therefore it is not an after-potential in the sense of negative after-potentials in nerve.

(c) Velocity of propagation. Both spike and slow wave are propagated, as seen from their diphasic form when both leads are on normal muscle. As the conduction distance in-

creases, the spike and slow wave become more separated, and velocities can be obtained by varying the conduction distance (fig. 1). Averages of velocities for 7 sets of measurements of conduction of the spike from anterior to posterior end varied from 0.9 to 2.2 m.p.s. (mean 1.09) and from posterior to anterior end averages of 5 preparations were from 1.0 to 1.9 m.p.s. (mean 1.4). The average velocity of all measurements on 14 preparations was 1.6 m.p.s. The most reliable measurements appear to be slightly faster than this average. When the muscle was stretched, velocity remained unchanged. The velocities for the slow wave averaged in 7 preparations 0.34 m.p.s. with the most reliable being slightly slower. The measured velocity of the slow wave was independent of direction.

It is concluded that the spike is conducted 5 to 8 times faster than the slow wave, that the velocity of each is the same in either direction in the muscle, but that both are propagated and hence the slow wave is not similar to an end-plate potential.

(d) Fatigue and facilitation. When the muscle was stimulated repetitively, the spike and slow wave behaved in opposite manners. The spike became reduced but usually continued at a low level at frequencies of 1 to 5 per second. At frequencies of 5 to 20 per second the spike rapidly became reduced in height and disappeared entirely after from 5 to 12 impulses (figs. 2 and 3). At higher frequencies the spike followed for a few stimuli, even at 60 per second, and then disappeared. The rate of fatigue of the spike varied among preparations and seemed to depend on the state of the muscle, particularly on the length of time since prior stimulation.

The slow wave, however, increases on repetitive stimulation (figs. 2 and 3). At stimulus intensities and amplifications at which the slow wave was not seen after single stimuli, it appeared and built up to a maximum after several stimuli. At frequencies of 10 per second and lower, repolarization was

incomplete and at frequencies above 30 per second ripples appeared on a maintained level of depolarization. Fusion of the electrical response was complete at about 60 per second (figs. 2 and 3).

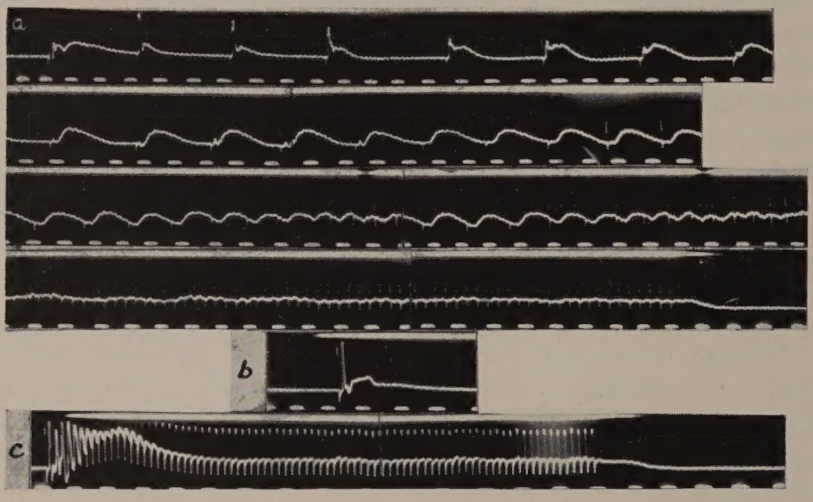

Fig. 2 Phascolosoma muscle. (a) Action potential series at gradually increasing frequency, showing decline of spike, increase in slow wave and negative retention. (b) Response to single stimulus. (c) Response at 60/sec. showing rise and decline of slow wave and negative retention. Time record 0.1 sec.

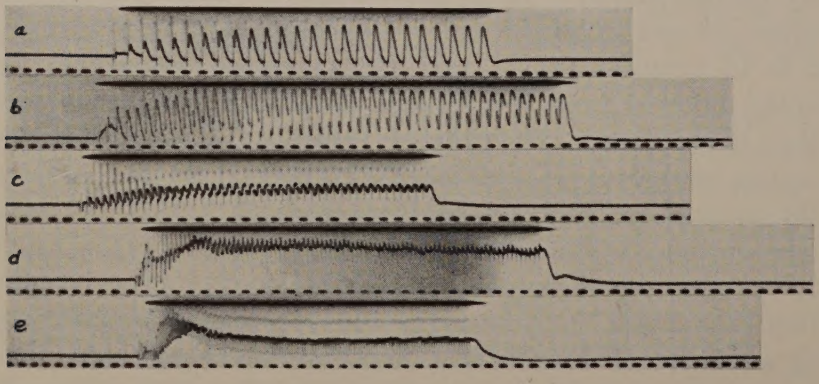

Fig. 3 Phascolosoma muscle. Action potentials at (a) 10/sec., (b) 15/sec., (c) 20/sec., (d) 40/sec., (e) 60/sec. Time record 0.1 sec.

After a tetanus there might be some negative retention, repolarization requiring severals tenths of a second (figs. 2 c and 3 d, e).

After prolonged repetitive stimulation the slow wave became reduced, but it showed much greater persistence than the spike response. The summed slow wave exceeded by several times the height of separate slow waves and sometimes also exceeded that of the spike.

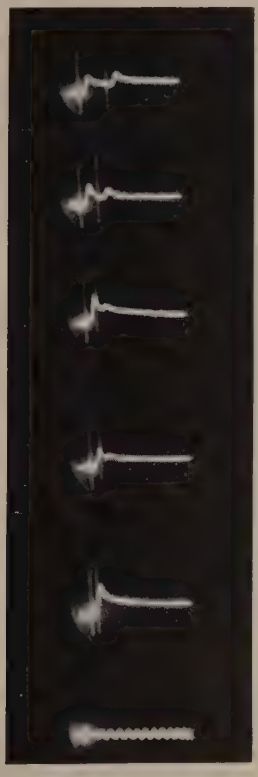

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Fig. 4 Phascolosoma muscle. Paired stimuli at decreasing intervals at intensity eliciting spike only, showing relative and absolute refractoriness. Time 60/sec.

Fig. 5 Phascolosoma muscle. Paired stimuli at decreasing intervals at intensity eliciting both spike and slow wave, showing refractoriness of spike and apparent absence of refractoriness of slow wave. First and last responses to single shocks. Time 60/sec.

(e) Refractory period. When pairs of stimuli at varying intervals were applied to the muscle, the spike response to the second shock showed some reduction at about 50 msec. interval (fig. 4). Refractoriness increased as the interval shortened and a spike falling in the descending phase of a prior one was very small. No second spike occurred to stimuli at the peak or during the rising phase, the absolute refractory periods being about 15 msec. The spike, like other propagated, all-or-none responses, shows relative and absolute refractoriness.

The slow wave, on the contrary, increased at all intervals of paired stimuli out to separations of several tenths of a second. Even a second stimulus falling in the rising phase of response elicited an enhanced slow wave (fig. 5). Thus it appears that the slow wave shows only facilitation and no apparent refractory period as if the response occurred in parallel elements requiring different amounts of summation.

(f) Excitation constants. Several strength-duration curves were obtained using the action potentials as end-points. Accurate thresholds for the slow wave were difficult to obtain, but the strength-duration curve of the slow wave lies well above and to the right of that of the spike. Chronaxies of the order of 1 to 3 msec. for the spike and 2.5 to 6 msec. for the slow wave were obtained.

(g) Effects of potassium. Potassium is a very effective stimulant for the Phascolosoma muscle. When a drop of potassium chloride (2%) was moved along the muscle which was in oil there was general contraction. When the potassium was applied at one region a contraction was seen in that region only. No action potentials were observed to accompany the potassium-induced contractions. When a drop of isotonic KCl was left at a point on the muscle, the action potentials in that region became reduced and in a minute or two conduction of the spike was blocked while the slow wave continued to pass. Ultimately the slow wave also was reduced but local application of KCl was a convenient method of obtaining

pure slow wave responses. The conduction mechanism of the spike is, therefore, more sensitive to KCl than is the conduction of the slow wave.

(h) Effects of injury and degeneration. Local injury was sometimes evident by the appearance of a white region in the muscle. Injury resulted in a rise in threshold, particularly of the spike response. Conduction of the spike could be blocked by local injury while the slow wave might be conducted through such a region. Sufficient injury, however, blocked the slow wave also.

Several muscles were placed in sea water in a refrigerator for several days. When tested at two and three days both spike and slow wave responses were normal, at 8 days both were gone and there was no visible contraction. At 5 days one preparation gave no response, three gave slow waves but none gave a spike. Apparently then, both conduction systems persist for at least three days after denervation and the slow system lasts longer than the spike.

II. Injury potentials. When a drop of isotonic KCl was added at the distal recording electrode, the action potentials became less diphasic and sometimes became strictly monophasic. When the end was crushed, a similar effect resulted and repolarization after an impulse was slower than with KCl at the end of the muscle.

The resting potential was measured from a pinched end to various points along the muscle. The following values were obtained in 5 preparations for distances of approximately 1 cm:

RESTING POTENTIAL	SPIKE POTENTIAL
mv.	*mv.*
2.3	
2.8	1.5
2.1	2.75
1.3	2.2
2.4	3.1

The resting potential increased with distance from the crushed end.

These results indicate that there is electrical (protoplasmic) continuity along the muscle.

III. Contractions as correlated with action potentials. (a) Properties of the contraction. Contractions recorded electronically by means of an isometric lever system showed contraction times of about 50 msec. and time for half relaxation 300 to 900 msec.

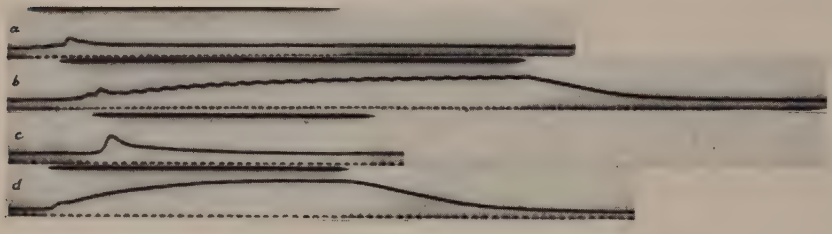

Fig. 6 Phascolosoma muscle. Contractions (a, b) at 5/sec., (c, d) at 20/sec., (a, c) at low intensity which elicited spike only, (b, d) at higher intensity which elicited spike and slow wave. Time 0.1 sec.

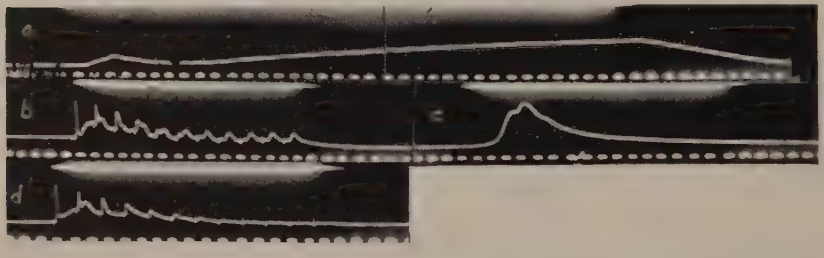

Fig. 7 Phascolosoma muscle, (a) contraction and (b) action potentials at 9.6/sec., at intensity eliciting both spike and slow wave; (c) contraction and (d) action potential at 9.6/sec. at intensity eliciting spike only. Time 0.1 sec.

The Phascolosoma muscle is very extensible and can easily be stretched to several times its "resting" length. As the muscle was stretched, tension in response to stimulation increased to a maximum and then decreased.

When the distance between stimulating electrodes was varied, the tension was similar at all separations. That is, the contraction was not restricted to the region between stimu-

lating electrodes but rather resulted from a propagated response.

(c) Correlations with potentials. When stimulated at such an intensity that only spike responses were elicited, the contractions were quick and not maintained (fig. 6 a, c; fig. 7 c, d). When the intensity was then increased to bring in both spikes and slow wave the mechanical response was initially quick, then it often diminished slightly and rose to a maintained level (fig. 6 b, d; fig. 7 a, b). When the only action potential was the slow wave, as when the spike was blocked by local KCl or by local injury or when the threshhold of the slow wave was lower, the rate of rise of tension was slow but the peak tension was similar to that when both spike and slow wave were seen. When only the slow wave was present, the contraction required more stimuli, hence more summation, than when there were spikes also. The correlation of slower contraction and longer maintained tension with the slow potentials was more evident with repetitive stimuli at intermediate frequencies than with widely separated shocks.

Some preparations showed a delay of repolarization lasting for several tenths of a second after a tetanus (fig. 2 c), and it was considered possible that slow relaxation might be correlated with this negative retention as in heart muscle (Curtis, '49). However, the relaxation time usually was several times longer and slow relaxation was observed in preparations showing little residual negativity.

Twitches are distinctly separate at frequencies below 10 per second, separate contractions can be detected during the rise but not during the plateau of contraction at 10 to 20 per second and at about 20 per second fusion is complete (fig. 8). The slow potential fuses electrically at about 60 per second, hence the fusion of contraction is mechanical.

The tension of a tetanus was 3 to 10 times greater than that of a single twitch and the rate of increase of peak tension with frequency of stimuli was greater at low intensities than at high intensities. Tension-frequency curves showed much

variability in the frequency for maximum tension. The most reliable curves show a maximum between 20 and 40 stimuli per second. The lowest frequency for maximum tension is not correlated with any maximum in the slow potential. Also the maximum tension occurs at frequencies above frequency of mechanical fusion. Hence, maximum tension is not at the frequency where relaxation of parallel elastic elements is just not permitted, as it seems to be for vertebrate skeletal muscle.

IV. Stimulation of nerve and ganglion. The innervation of the proboscis retractor by a ganglion which lies imbedded in its insertion makes it impossible to isolate postganglionic

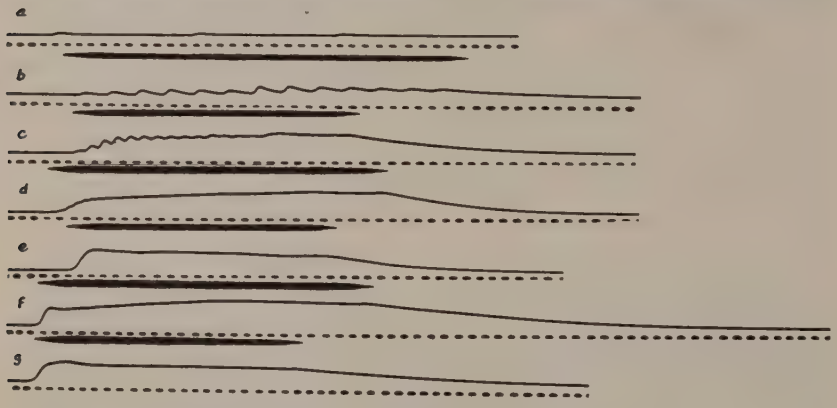

Fig. 8 Phascolosoma muscle, contractions at (a) 1/sec., (b) 5/sec., (c) 10/sec., (d) 15/sec., (e) 30/sec., (f) 60/sec., (g) 100/sec. Time 0.1 sec.

nerve fibers for stimulation. That the muscle is not innervated from its origin on the body wall was shown by complete absence of response when the central nervous system was stimulated after severing the esophageal connections.

Stimuli were applied to a preganglionic nerve freed from the esophagus and occasionally to the ganglion. The threshold of the nerve was consistently higher than of the muscle stimulated directly. The response to a single shock applied to the nerve or ganglion was a volley of spikes (fig. 9). Pinching or applying isotonic KCl in the region of the ganglion abolished the repetitive response and thereafter stimuli at

the insertion of the muscle elicited single spikes as described above. When the nerve was stimulated, the spikes fatigued much faster than when the muscle was stimulated and the number of spikes per volley decreased in successive responses at intervals of two to three seconds (fig. 9). Slow waves were also seen and after fatigue of the spike response, multiple

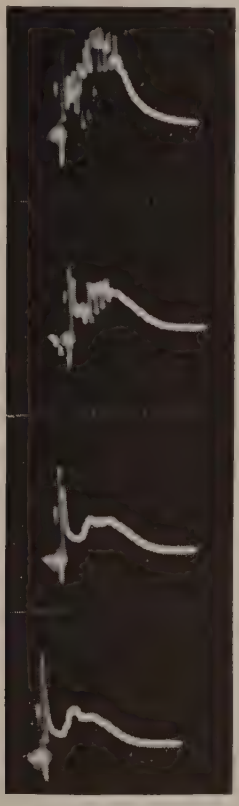

Fig. 9 Phascolosoma muscle, series of responses to single shocks applied to cephalic ganglion showing repetitive firing.

slow waves were sometimes noted. However these fatigued faster than with muscle stimulation. Spikes sometimes appeared only in the first response of a series and slow waves continued. It appears that the ganglion fatigues much more rapidly than the muscle.

It is probable that when the worm retracts its proboscis it sends out a volley of spikes which initiate a quick contraction and then cease. These spikes are replaced by facilitating and more persistent slow waves which tend to keep the muscle contracted. The muscle must remain actively contracted for long times when the proboscis is retracted during periods of high hydrostatic pressure in the coelom, pressure which would tend toward eversion of the proboscis.

V. Histological observations. Fixed muscles were examined in section and in macerated mounts. Single fibers from relaxed muscles are unbranched, spindle-shaped, 1 to 2 mm long and 3–6 μ in diameter as stated by Olson ('40). When contracted, both fixed and living muscles show a remarkable zigzag pattern of fibers with crests and troughs of many fibers aligned across the muscle. This pattern diminishes when the muscle is stretched and appears reversibly when the muscle contracts or shortens, hence the bands are not contraction nodes. It was impossible to separate by teasing or maceration the single fibers from contracted muscles. The fibers must be connected in some way so that they fold at corresponding points across the entire muscle; however observation of both teased and sectioned muscles failed to reveal connecting strands.

In several muscles lateral incisions were made on alternate sides and a contraction was observed to pass around the incisions. Also muscles were slit for a distance of about 1 cm at one end; stimulation of one of the resulting arms of the Y-shaped muscle resulted in what appeared to be uniform contraction in the basal part of the muscle and some contraction in the other arm. These observations are best interpreted by assuming a functional syncytium of muscle fibers. Also the evidence from injury and action potentials indicates a functional continuity throughout the muscle.

Lantern retractor muscles of Thyone

The lantern retractors of Thyone resemble the proboscis retractors of Phascolosoma in being very extensible non-

striated muscles. However they are very fragile and tend to pull apart when caused to contract under slight tension. Best results were obtained by leaving the muscle attached to a lantern tooth and tying rather loosely the origin at the body wall.

I. Action potentials. (a) Form and velocity. Both fast and slow components are shown in figure 10. The spike is more graded with intensity than is the spike in Phascolosoma and the spike is frequently multiple. The slow wave is less prominent than in Phascolosoma, both potentials are smaller, and quantitative data regarding the slow wave were difficult to obtain.

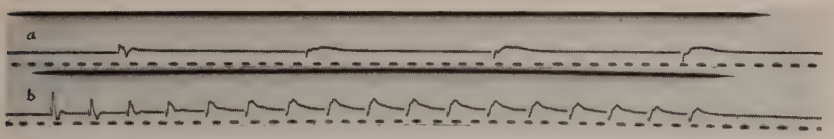

Fig. 10 Thyone lantern muscle, action potentials at (a) 1/sec., (b) 5/sec. Both fast and slow waves. Time 0.1 sec.

Both spike and slow wave were recorded monophasically and diphasically. Velocity measurements were readily made with the spike and the average of 9 measurements on 3 preparations was 0.26 m per second (limits 0.19–0.38).

(b) Responses to repetitive stimulation. At frequencies of 2 to 5 per second the spike response declined in height and disappeared completely after about 5 to 10 impulses (fig. 10). At the same time the slow wave facilitated somewhat. At higher frequencies of 5 to 10 per second decline of the spike was faster while the slow wave remained.

When paired shocks were applied the second spike was absent at stimulus intervals of 50 msec. and was reduced for as much as 500 msec. after the first stimulus.

II. Contractions. The contraction time for a single twitch was 300 msec. and half-relaxation time one to one and one-half seconds. The tension increased with separation of the stimulating electrodes; for example in one preparation tension cor-

sponded to 2 mm deflection on the oscilloscope at electrode separation of 2 mm and was 19.9 mm deflection at 10 mm separation. Mechanical fusion was complete at 5 per second.

It appears from these limited observations that the lantern retractors have two action potentials, comparable to those of Phascolosoma proboscis retractors. In Thyone the slow wave cannot always be obtained, the spike is more prominent. Also Thyone differs in the variation in spike height with distance between stimulating and recording electrodes and in variation in contraction with electrode separation. Apparently the fibers of the lantern muscles are shorter and are not continuous in the functional sense. As reported by Olson ('38), there is much more connective tissue between the muscle fibers than in the Phascolosoma muscles.

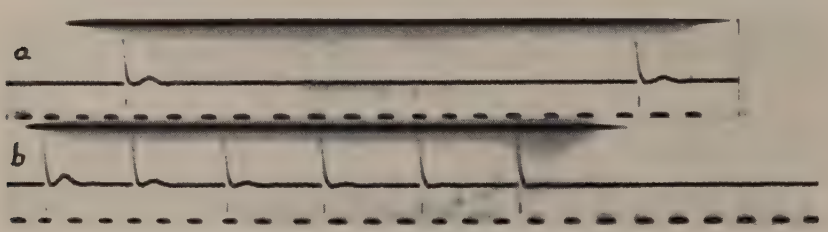

Fig. 11 Thyone long body wall muscle, action potentials at (a) 0.6/sec. and (b) 3/sec. Time 0.1 sec.

Longitudinal muscles of body wall of Thyone

The long body wall muscles of Thyone are much stronger and more resistant to stretching and manipulation than the lantern muscles. However they are less extensible and remain in a more contracted state after being cut free from the body wall.

(a) Action potentials. Many unsuccessful attempts were made to record potentials from the long body wall muscles. The muscle could be seen to contract between the stimulating electrodes but no action potentials could be recorded at a distance. However in 6 favorable preparations action potentials were obtained with the active recording electrode very

close to the stimulating cathode (fig. 11 a). Responses decreased with distance, however, so that none could be obtained at from 2 to 5 mm from the cathode. The response was more diffuse at the greater distance. In three sets of measurements velocity averaged 0.36 m per second.

On repetition the action potentials declined rapidly, even with widely separated shocks (fig. 11). At two and nine-tenths shocks per second the response disappeared after 3 or 4 stimuli. There was no evidence of a slow wave and from its tendency to fatigue and its greater diffuseness as conduction distance increased the only response recorded from the long body wall muscles appears analogous to the spike of Phascolosoma.

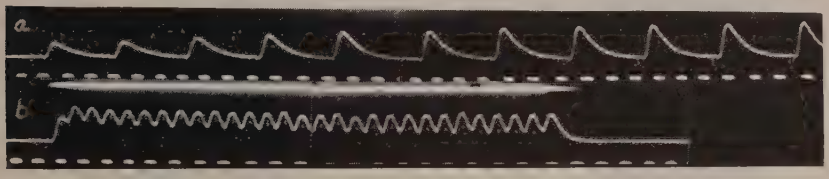

Fig. 12 Mytilus byssus retractor. Action potentials at (a) 3.5/sec. and (b) 15/sec. Time 0.1 sec.

(b) Contractions. Contractions in response to single shocks showed a contraction time of six-tenths to one second and a time for half-relaxation of two and two-tenths to several seconds. The tension increased greatly with increasing electrode separation, and contraction appeared restricted to the region between stimulating electrodes, for example, at 4 mm separation the tension corresponded to 4 divisions on the oscilloscope, at 14 mm separation it was 10 divisions and at 17 mm 17 divisions.

Byssus retractor of Mytilus

The action potentials of the byssus retractor of Mytilus have been studied in detail by Fletcher ('36) and the mechanical properties by Winton ('37) and others. Our observations add little to previously published data. Injury potentials of 5

to 6 mv. were obtained and action potentials were conducted the entire length of the muscle at an average velocity of 0.6 m per second.

When stimulated repetitively the action potential remained fairly constant in height at 0.8 per second, built up slowly

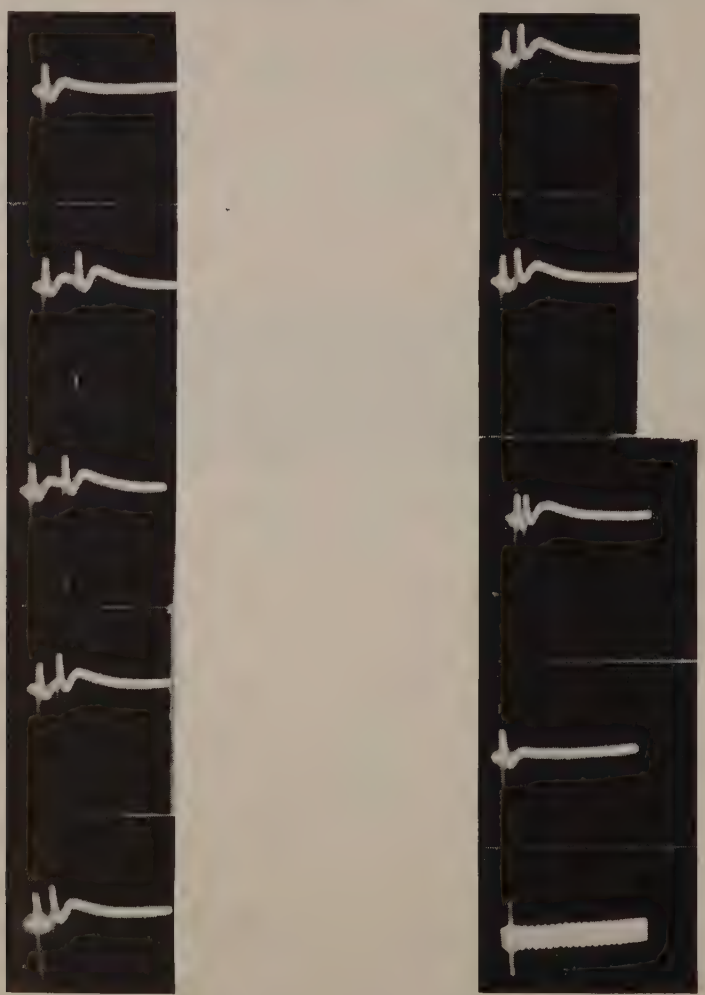

Fig. 13 Mytilus byssus retractor. Action potentials, paired stimuli at decreasing intervals showing apparent absence of refractoriness; first and last records are responses to single shocks. Time 60/sec.

at 2.8 and rapidly at 15 per second (fig. 12). When paired shocks were applied, the response to the second stimulus was larger than to one stimulus at all intervals, that is, facilitation prevented the appearance of a refractory period (fig. 13).

The electrical response of the byssus retractor of Mytilus appears to be analogous to the slow wave in the Phascolosoma proboscis retractor in that it shows summation on repeated stimulation, it does not fatigue readily and shows no apparent refractory period. It differs in that summation is less than in Phascolosoma but in both there can be complete fusion of the electrical response.

CONCLUSIONS

The properties of the spike and slow wave in Phascolosoma proboscis retractors and their apparent distribution in the muscles are summarized as follows:

SPIKE	SLOW WAVE
fast propagation	slow velocity
usually lower threshold	
shorter chronaxie	
fatigues rapidly	fatigues very little
no facilitation	much facilitation on repetitive stimulus
readily blocked by KCl and injury	not readily abolished
relative and absolute refractory periods	no apparent refractory period probably because of facilitation
multiple with greater separation of recording electrodes	becomes diffuse with distance from stimulus
associated with quick transient contraction	associated with slow maintained contraction
present in Phascolosoma, in Thyone lantern and body wall retractors	present in Phascolosoma, in Thyone lantern retr. and Mytilus byssus retr.

Evidence is not conclusive as to whether the muscles are being stimulated directly or by nerve elements in them. Certainly the recorded potentials are of such magnitude that they must be muscle action potentials. The simplest explanation of the preceding observations in Phascolosoma would be that

the muscle receives two kinds of nerve fiber, one which elicits a propagated fast response and the other a graded, slow, end-plate type potential as in crustacean muscle. However, there is the following evidence against nerve stimulation: (1) the nerve threshold in Phascolosoma was consistently higher than the muscle threshold, (2) responses were identical whether the muscle was stimulated from innervated or non-innervated ends, (3) the spike response persisted for three days and the slow wave 5 days in denervated preparations in a refrigerator. Against the suggestion that the slow wave is an end-plate potential is the following: (1) the slow wave is propagated at a measurable velocity, (2) no foci of origin could be found, (3) the slow wave becomes diffuse as conduction distance increases.

No histological differences among the muscle fibers could be seen, yet the conduction systems can be separated by threshold, effects of KCl, fatigue, injury and degeneration. Whether both types of potential occur in the same fibers is not known; analysis in terms of single fibers will be very difficult, if not impossible. Functional continuity throughout the Phascolosoma muscle is indicated by (1) injury potentials, (2) synchronous action potentials which can be either monophasic or diphasic according to recording conditions, (3) conduction around incisions, (4) conduction throughout the muscle. Yet histologically the isolated elements are short spindle-shaped smooth fibers. There must be functional continuity from fiber to fiber even though this has not been demonstrated histologically. The fast and slow potentials elicit quick and maintained contractions respectively, yet observations of contracting muscles failed to show any localized contractions under various conditions.

By the criteria presented above both types of potential were found in two kinds of non-striated muscles, the spike only in one, and the slow wave only in another. Both fast and slow potentials have been described from numerous smooth muscles; it is possible that their properties are similar to those

described in these relatively simple preparations. The potentials described above may constitute the electrical basis for the differences between phasic and tonic contractions in non-striated muscle.

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THE LOCAL RESPONSES OF MAMMALIAN SPINAL ROOTS TO ALTERNATING SINUSOIDAL CURRENTS

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THIRTEEN FIGURES

In earlier communications from this department (Rosenblueth and Luco, '50; Rosenblueth and García Ramos, '51) observations were presented on the local responses of myelinated mammalian axons to anodal and cathodal rectangular pulses.

The present study of the responses to subthreshold sinusoidal alternating currents was undertaken because of the intrinsic importance of the problem and because the method employed by Rosenblueth and García Ramos for the recording of the responses is quite suitable for this study.

METHOD

The nerves used were spinal roots, removed from cats anesthetized with dial (0.65 cm^3 per kg, intraperitoneally). They were placed on non-polarizable electrodes and immersed in mineral oil, at room temperature (20 to 25°C.).

The detailed description of the electrodes and of the recording apparatus may be found in the paper by Rosenblueth and García Ramos ('51).

The alternating currents (a.c.), of variable frequency, were generated by a Hewlett-Packard, 200-DR, audio-oscillator. They were led to the primary of a transformer whose output voltage was constant within the range of frequencies employed (from 7 to 2,000 cycles per sec.). A potentiometer

placed across the secondary of the transformer controlled the amplitude of the currents delivered to the nerves.

RESULTS

A. The balancing of the currents applied. The method used for minimizing in the records the passive changes of potential elicited by the impressed e.m.f. was described and

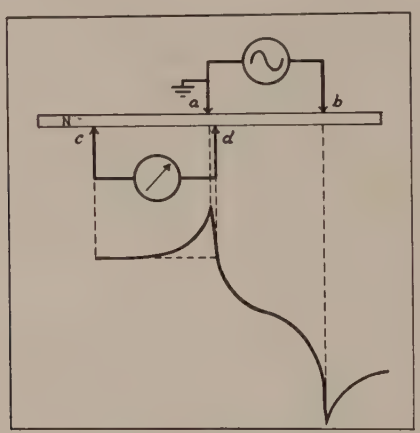

Fig. 1 Diagram of the method employed to minimize in the records the passive electrotonic component of the a.c. applied. At any moment, when current is flowing through electrodes *a* and *b*, the distribution of potential along the nerve will be qualitatively as shown by the lower curve, with the appropriate sign. By moving electrode *d* along the nerve it is possible, therefore, to find a position in the interpolar region, that will make this electrode isopotential with the other recording electrode *c*. As electrode *d* crosses the position of optimum balance the sinusoidal recorded will change sign, i.e., there will be a phase shift of 180°.

In the following figures the position of the recording electrodes will be described by referring to the letters in this diagram.

discussed in detail by Rosenblueth and García Ramos ('51). As shown in the diagram of figure 1 this method consists essentially in moving an electrode (*d*) near one of the poles (*a*) within the interpolar region, until it becomes as isopotential as possible to another distant recording electrode during the passage of current. Theoretically, if the nerve behaved like a passive linear conductor a perfect balance should be

obtained, i.e., the a.c. should be entirely canceled in the records if electrode *d* is at the proper position. In practice the balance was not perfect, however, even at the optimum position. It was better for low than for high frequencies of a.c. In addition the optimum position shifted when the frequency was changed. This shift is due to the change of the impedance of the nerve that corresponds to a variation of the frequency. The absence of a perfect balance for any given frequency

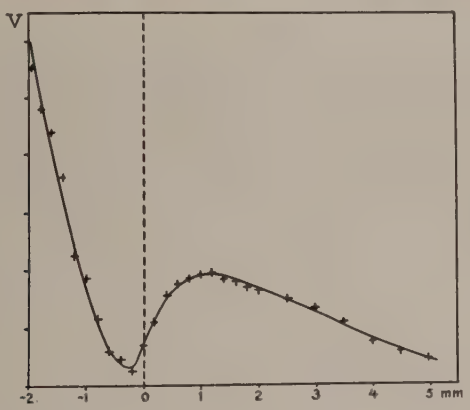

Fig. 2 Electrotonic distributon of a.c. along the lower edge of a spinal root in the vicinity of the overlying pole. A.c. with frequency 80 per sec. and with an intensity approximately 0.1 threshold was applied, and electrode *d* was moved along the nerve. Abscissae: distances from the point opposite the pole, with the extrapolar segment counted as positive. Ordinates: peak to peak amplitude of the sinusoidals recorded, in arbitrary units. The potential changes showed a phase shift of 180° between 0 and — 0.3 mm.

is in turn due to the fact that the impedance is not linear but changes with the intensity of the current and with the direction of the flow.

It was always easy to locate the critical region for electrode *d* by moving it along the nerve with a micrometer. It was usually about 0.3 mm from the pole, and shifts of less than 0.2 mm in either direction resulted in marked unbalance (see fig. 2). With frequencies of less than about 500 per sec. it was always possible to obtain a degree of balance that allowed the ready recognition of the local responses.

B. Criteria for the identification of the local responses. If a.c. of a given frequency, e.g. 70 per sec., is applied with increasing intensity, and records are taken with the electrode *d* of the diagram in figure 1 at or near the optimum position for balance, the following changes are observed. At first an almost pure sine wave is recorded (fig. 3 A and B), which is usually out of phase with the impressed e.m.f. (fig. 3 H). As the intensity augments, the sinusoidal tracing grows and becomes more distorted by the regular superimposition of an

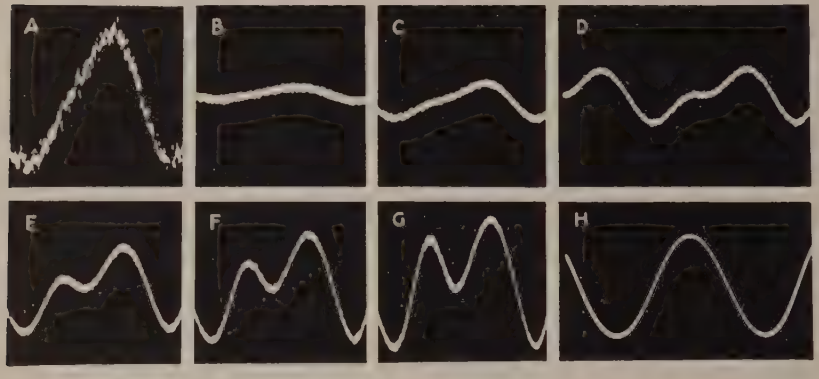

Fig. 3 Changes in the records when the intensity of the a.c. is varied. Electrode *d* at the position of optimum balance. B to G: a.c. (100 cycles per sec.) applied with gradually greater intensity; in G it was just below threshold. A: as in B but with 10 times greater amplification. H: record from the pole, to show the phase relations (cf. wth D).

additional wave, which grows in a nonlinear manner until the threshold for spike discharge is reached (fig. 3 C to G).

The distorting additional wave denotes a local potential change, for it does not appear in records taken with electrode *d* placed 7 to 10 mm away from *a*, in the extrapolar region.

The application of single maximal brief shocks in the course of a.c. subthreshold stimulation results in the decrease or disappearance of the additional wave. Figure 4 shows a characteristic example. It is clear that in the cycles that follow the spike discharge elicited by the brief shock, the additional

wave is considerably reduced, whereas the sinusoidal component is practically unaffected. It can therefore be inferred that the refractory state opposes the development of the wave under consideration.

The additional wave also disappears if the nerve is blocked by applications of strong a.c. Figure 5 illustrates a typical instance. After the passage of strong, high-frequency current, in the records with weak 100-cycle current the sinu-

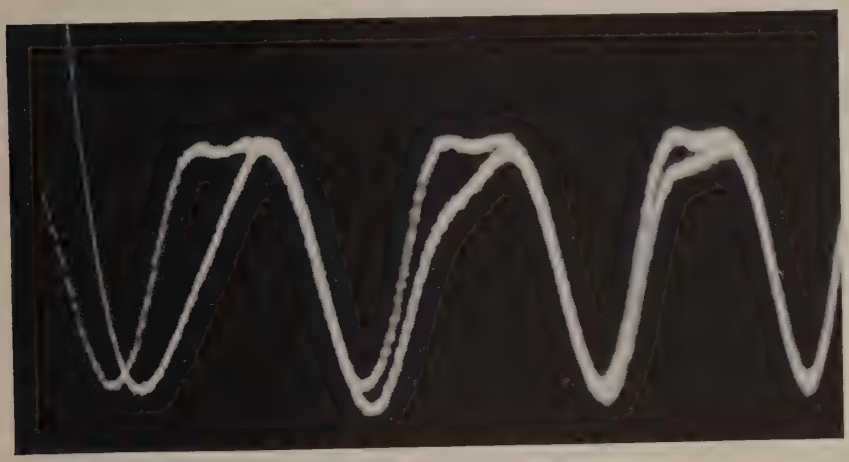

Fig. 4 Decrease of the local responses during the refractory period. A.c. (300 cycles per sec.) was applied to electrodes *a* and *b*. Through the same electrodes a brief maximal d.c. pulse was delivered about 30 times per sec. The figure shows two sweeps. One is a control, with uniform local responses. The other begins with the end of the maximal spike elicited by a brief pulse; the local response is almost absent in the following cycle and recovers gradually thereafter.

soidal component was greater than originally, thus showing an unbalance due probably to a local change of impedance. This component gradually decreased, as the nerve recovered. The additional wave, which had disappeared entirely, did not begin to be identifiable until well after the sinusoidal component had decreased to its original amplitude. It thereafter grew, and the final record is similar to the original control. It can therefore be inferred that the additional wave develops only in nerves that are functionally normal.

It is thus legitimate to analogize the additional local component in the records obtained upon applications of a.c. with the local responses elicited by rectangular pulses, studied by Hodgkin ('38), Arvanitaki ('39), Pumphrey, Schmitt and Young ('40), Katz ('47), Rosenblueth and Luco ('50), and Rosenblueth and García Ramos ('51). Consequently, we shall hereafter designate that component as the local response to a.c. stimulation.

C. The measurement of the local responses. The observations described above lead to the conclusion that the rec-

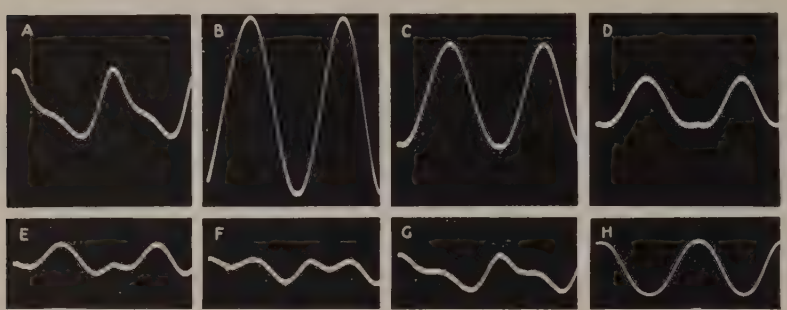

Fig. 5 Reversible disappearance of the local responses after blocking the nerve with strong a.c. A, control, with electrode *d* near the position of optimum balance; responses to a.c. (100 cycles per sec.). B to G, as in A, at approximately 1 min. intervals after the application of strong, high frequency (10,000 cycles per sec.) a.c. for 30 sec. This application resulted in the transient unbalance of the records. The local responses, which had disappeared (B and C), gradually reappear. H, record from the pole, with less amplification.

ords such as those illustrated in figure 3 represent the summation of a passive sinusoidal component and a local response. On the basis of this conclusion it is easy to estimate the time course and amplitude of the local responses. The method is illustrated in figure 6 for several frequencies of a.c. A two-beam Electronic Tube Corporation oscillograph was used. One of the beams recorded the local changes; the other traced a sinusoidal adjusted in phase, time-scale and amplitude so as to fit the segments of the record interpreted to correspond to the passive component. The difference between the two

tracings indicated the amplitude of the local response at any given instant (see table 1).

In practice we usually reconstructed the local responses as follows. The records were taken as in figure 3. They were projected through a photographic enlarger and drawn on paper. A sinusoidal curve was then selected, out of a series with different amplitudes and appropriate time scales, that would fit accurately the passive component of the records

TABLE 1

Ang°.	F10	F40	F50	F70	F150	F250	F500	F800	Av.	Av. (25)
—132	0	0	0	0	0	0	0	0	0	0.1
—120	0	3.1	0	0	0.4	0	1.9	0	0.7	1.0
—108	0.6	9.4	3.2	1.6	3.6	0.6	9.3	2.0	3.8	3.1
—96	2.3	14.1	11.7	11.3	8.5	2.8	16.7	5.8	9.2	8.5
—84	8.3	25.0	17.0	19.4	14.2	13.9	25.9	9.6	16.7	14.8
—72	15.9	32.8	29.8	25.8	24.4	25.0	35.2	17.3	25.8	24.7
—60	27.3	43.8	39.4	33.9	36.2	44.4	48.1	35.8	38.6	37.8
—48	43.2	64.1	53.2	50.0	49.7	58.3	61.1	47.6	53.4	53.3
—36	61.5	79.7	64.9	67.7	65.5	75.0	74.1	65.4	69.2	69.9
—24	75.0	87.5	80.9	87.1	84.2	88.9	87.0	80.3	83.9	84.5
—12	91.0	93.8	94.7	96.8	97.3	94.4	96.3	94.2	94.8	95.6
0	100	100	100	100	100	100	100	100	100	100
12	91.0	97.0	96.8	96.8	96.3	91.7	92.6	93.8	94.5	95.7
24	84.1	90.6	93.6	93.5	77.6	75.0	75.9	80.3	83.8	85.0
36	68.2	73.4	80.9	77.4	55.3	58.3	53.7	61.5	66.1	69.6
48	59.1	57.8	61.7	59.7	41.9	38.9	33.3	36.5	48.6	52.7
72	50.0	46.9	45.8	40.3	29.3	22.2	20.4	15.4	33.8	36.5
84	36.4	31.3	34.0	24.2	16.3	13.9	11.1	6.7	21.7	22.7
96	25.0	21.9	23.4	12.9	7.3	5.6	3.7	2.1	12.7	13.2
108	18.2	10.9	16.0	4.8	2.5	2.8	1.9	0.4	7.2	6.4
120	7.6	6.3	8.5	1.0	0.4	0.4	0	0	3.0	2.6
132	2.3	1.6	3.2	0	0	0	0	0	0.9	1.1
144	0.2	0	1.1	0	0	0	0	0	0.2	0.5
156	0	0	0	0	0	0	0	0	0	0

Amplitude of the local responses to different frequencies of a.c. at different times during the cycle. The column headed Ang°. gives the phase angles in degrees. The following 8 columns headed F– give the amplitudes for different frequencies of stimulation of a nerve. There was a phase shift, but it has been neglected by placing the peak at 0°. The amplitudes are calculated as percentages of this peak. The column headed Av. is the arithmetic mean of the corresponding rows, and that headed Av. (25) is the mean of 25 responses in three different nerves

when projected through the enlarger. This curve was drawn in the proper position on the corresponding tracing of the record. The difference between the two curves could then be measured readily. As a rule the records were taken with electrode *d* not at the position of optimum balance, but slightly

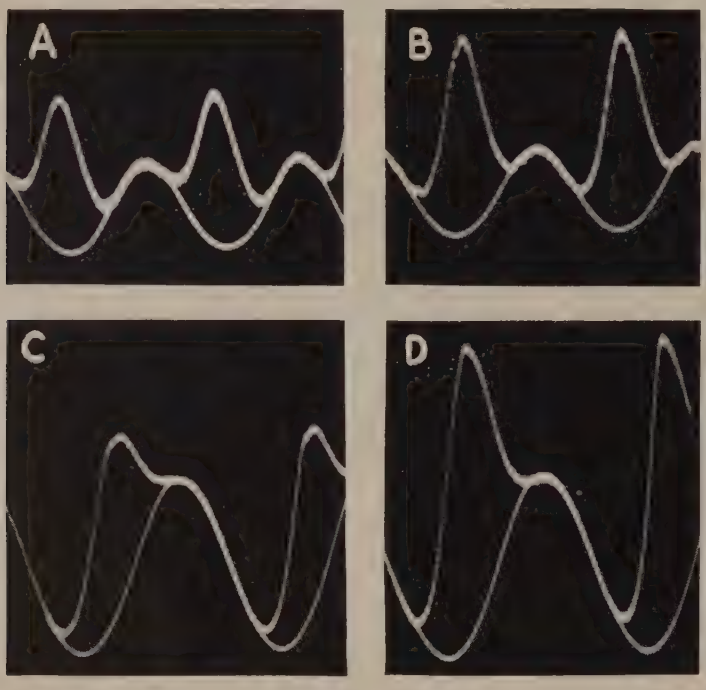

Fig. 6 Measurement of the local responses by the adjustment of a sinusoidal curve to the passive component of the records. A two-beam oscillograph was used; one beam recorded the responses, with electrode *d* slightly off balance; the other beam traced a sinusoidal curve which was adjusted in amplitude and phase to the passive component of the records.

A to D: 30, 50, 100 and 150 cycles per sec.

shifted toward the pole, in order to emphasize the passive component. This procedure made the matching with the sinusoidal standards more accurate and easy.

D. The influence of the intensity of the currents. Figure 7 shows the peak amplitude of the local responses to a.c. with

fixed frequency and variable intensity. As the current is intensified the amplitude increases, slowly at first and then more rapidly until the threshold for spike discharge is reached. The curve is similar to that which corresponds to rectangular cathodal or anodal pulses (see Arvanitaki, '39; Rosenblueth and Luco, '50; Rosenblueth and García Ramos, '51).

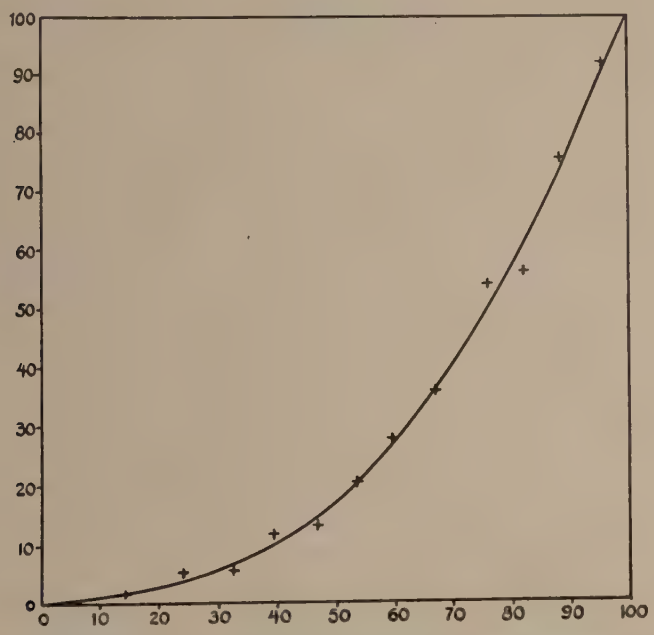

Fig. 7 Influence of the intensity of the current on the amplitude of the local responses, at equilibrium. Abscissae: intensity of the a.c. (150 cycles per sec.); 100 units was the just-threshold intensity. Ordinates: peak amplitude of the local responses (in arbitrary units).

E. The influence of the frequency of the currents. The local responses to currents with different frequencies vary in duration, in amplitude and in their phase relations to the impressed e.m.f. Figure 8 illustrates the changes in the amplitude of the local responses as a function of the frequency of the a.c. when the voltage is maintained constant.

As is well known, there is an optimum frequency for a.c. stimulation, at which the threshold for spike discharge is lowest; slower or faster frequencies require stronger currents. In some experiments the frequency was varied and the intensity was adjusted for each frequency so as to make it just threshold for the most excitable fibers in the nerve.

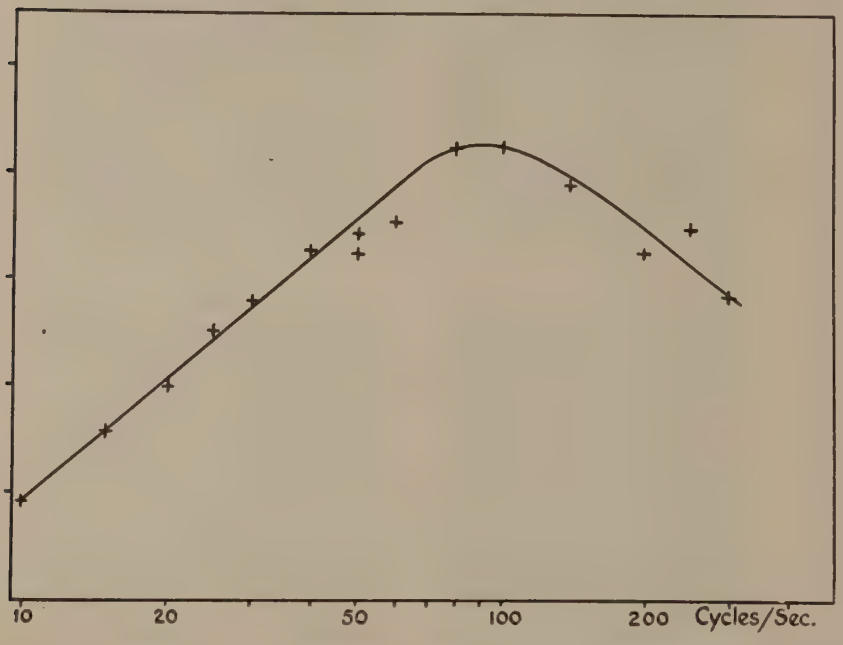

Fig. 8 Influence of the frequency of the a.c. on the amplitude of the local responses. Abscissae: frequency per sec. (logarithmic scale). Ordinates: peak amplitude of the responses. The intensity was maintained constant, and was just threshold at 90 cycles per sec.

Figure 9 shows the intensity of the threshold currents (upper curve) and the peak amplitude of the corresponding local responses (middle curve); the two curves are similar in shape. The areas of the threshold responses are shown in the lower curve; they are approximately constant for frequencies between 50 and 600 per sec.

F. The time course of the local responses. In figure 10 are illustrated the positions in the cycles (in degrees) of the beginning, peak and end of the local responses to a.c. of different frequencies, in a typical experiment. The intensity of the currents was just threshold for spike discharge at all

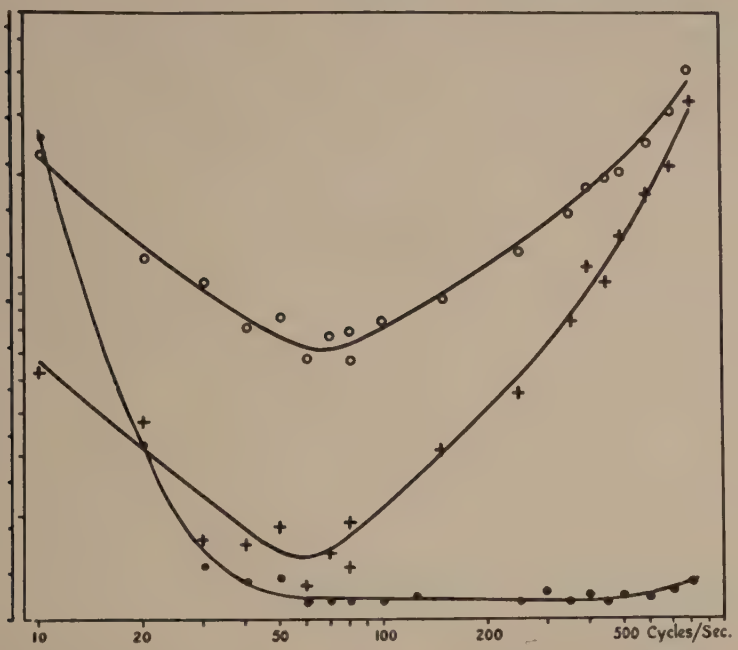

Fig. 9 Influence of the frequency of the a.c. on the characteristics of the just-threshold local responses. Abscissae: frequency per sec. (logarithmic scale). Right hand logarithmic scale of the ordinates: upper curve (circles), intensity of the threshold currents; middle curve (crosses), peak amplitude of the corresponding threshold local responses. Left hand arithmetic scale of the ordinates: lower curve (dots), areas of the same responses.

the frequencies. The duration and time course of the responses remain approximately constant if the duration of the cycle is taken as unit. On the other hand, there is a gradual shift of phase, the response lagging more and more with regard to the impressed voltage as the frequency increases. Since this lag is measured in degrees, that is, as a fraction of the cycle, its duration in msec. is not shown by the

lines in figure 10. If the durations are calculated, it is found that the lag, measured from the peak of the positive half-cycles, is practically zero up to a frequency of 20 per sec.; it then increases to reach a maximum of about 1.4 msec. at about 50 per sec., it thereafter decreases.

Table 1 shows the average time course of the local responses to different frequencies of a.c. of just-threshold intensity

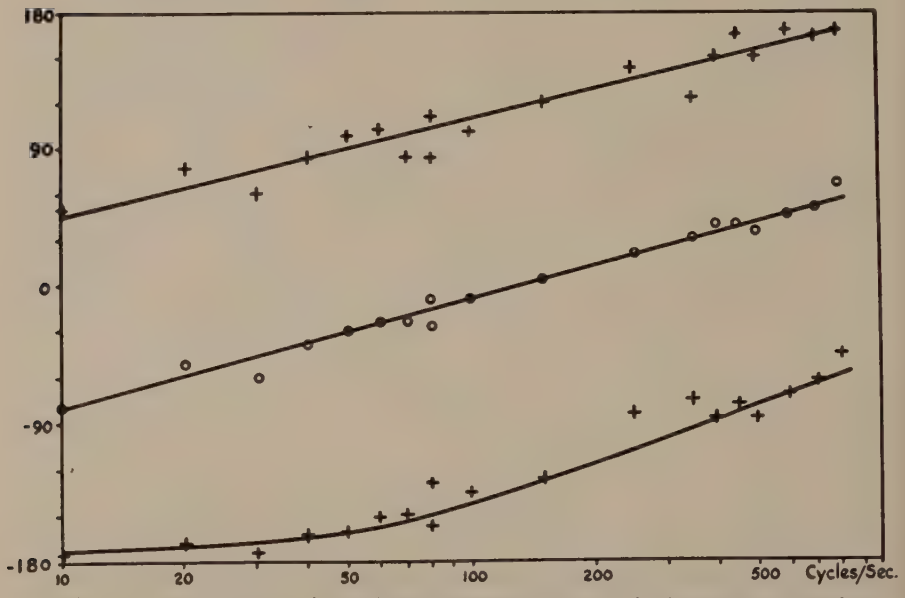

Fig. 10 Time course of the local responses to a.c. of different frequencies. Abscissae: frequency per sec. (logarithmic scale). Ordinates: time, measured in degrees per cycle, with the beginning of the cathodal half-cycle as zero. Lower, middle, and upper curves: beginning, peak, and end of the local responses, respectively.

in three nerves, the duration of the cycle being taken as unit of time and the peak amplitude of each response as 100 units of amplitude. Clearly a uniform law arises, which describes the time course of the responses, and which is the same for different frequencies in each nerve and for different nerves. When measured in the same way, the responses to different intensities of a.c. with a given frequency all yielded again

the same results, that is, they adjusted to the same general law.

The average local response in table 1 is shown in figure 11 by the crosses. The curve in this figure corresponds to the equation

$$R = 13.91\ (e^{2\cos(2\pi Ft + \phi - \theta)} - 0.2),$$

where R is the amplitude of the response measured as per cent of the maximum, F is the frequency, t the time in seconds, ϕ the phase angle of the starting time, and $\theta = 0.49 \log_e$

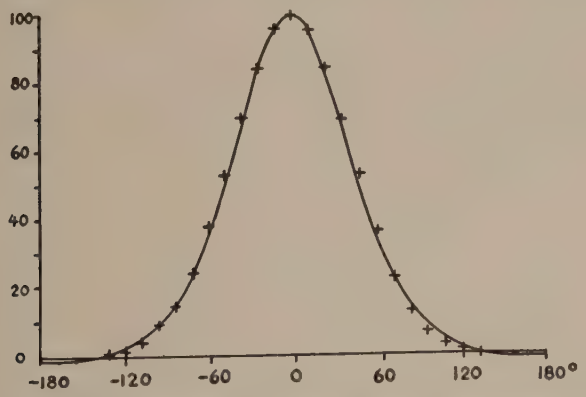

Fig. 11 Average local response. Abscissae: time in degrees, as in the ordinates of figure 10. Ordinates: amplitude of the response. The crosses are the average in table 1 (column Av. 25). The curve is the locus of an equation given in the text.

(F/20) is the phase shift, in radians, of the response with respect to the a.c., calculated from the data in figure 10.

The satisfactory agreement of the theoretical curve with the experimental data indicates that the equation used describes adequately the time course of the local responses to a.c. of any subthreshold intensity, within a range of frequencies between 20 to 800 per sec., and at a temperature of approximately 22°C. It is possible that this time course may be different at other temperatures and may also change in other nerves or in other experimental conditions. It is interesting to note that the response in figure 11 is symmetrical

with respect to the peak, as are those in table 1, even when there is a significant shift of phase with respect to the a.c. applied.

G. The distribution of the local responses along the nerve. Experiments similar to that illustrated in figure 2 were carried out with an intensity of the a.c. just threshold for spike

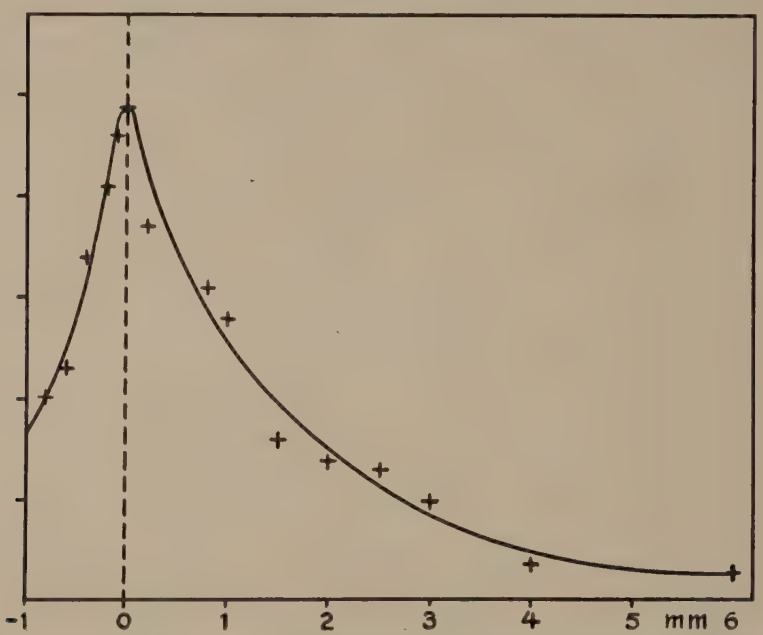

Fig. 12 Distribution of the local responses along the nerve in the vicinity of the pole. Abscissae as in figure 2. Ordinates: peak amplitude of the local responses to a.c. (80 cycles per sec.) at the corresponding positions of electrode *d*.

discharge of the most excitable fibers. The peak amplitude of the corresponding local responses was then measured at each of the different positions of electrode *d*.

Figure 12 shows a typical set of results. It is clear that the local response was greatest at the region just underlying the pole, and decreased markedly in amplitude at regions more distant from this pole. It is interesting to note that the distribution of the local response differs significantly from

that of the electrotonic spread of the a.c., when both are recorded with the same method (cf. figs. 2 and 12).

H. The initial responses to a train of alternating waves. The description has dealt so far with the responses seen at the steady state, i.e., some time after a given current begins to flow through the nerves. It was deemed important to observe the initial effects, and in particular to study the influence of the sign, cathodal or anodal, of the beginning of the flow, on those initial responses.

For this purpose a double-pole single-throw switch was used; one side tripped the sweep of the oscillograph and the other closed, within a few milliseconds, the circuit for the flow of a.c. through the primary of the transformer. The single sweep of the oscillograph beam thus obtained showed the effects of the first cycles of the a.c. The results were as follows.

Different frequencies, between 10 and 400 per sec., were selected, and the voltage was adjusted so that it was slightly below the threshold for spike discharge at the steady state. In these circumstances, if the current began to flow at the beginning of the cathodal half-cycle, the local response to this cathodal pulse was minimal (fig. 13 B); a clear response appeared only after the following anodal half-cycle began to subside, and reached its peak, as usual, shortly after the beginning of the subsequent cathodal wave. On the contrary, if the initial pulse of the current was anodal, a marked local response developed during the first cycle (fig. 13 A).

If the response to the first or second cycle was large, with frequencies higher than about 50 per sec., the following responses decreased or alternated. With high frequency a.c. (over 2,000 per sec.) the responses usually grew over a few cycles so that spike discharge occurred only after the 3rd or 4th response.

I. The timing of the discharge of spikes. When the local responses are sufficiently ample, a.c. evokes conducted impulses at each cycle. If the intensity of the a.c. is just threshold, that is, if only a few fibers discharge, the spikes

begin well after the local responses have reached their peak. This late discharge is similar to that seen with just-threshold cathodal shocks (Rosenblueth and Luco, '50).

As the a.c. is intensified the initiation of spikes occurs gradually earlier. When the stimuli activate about 30% of the fibers, the spikes begin before the peak of the corresponding local responses.

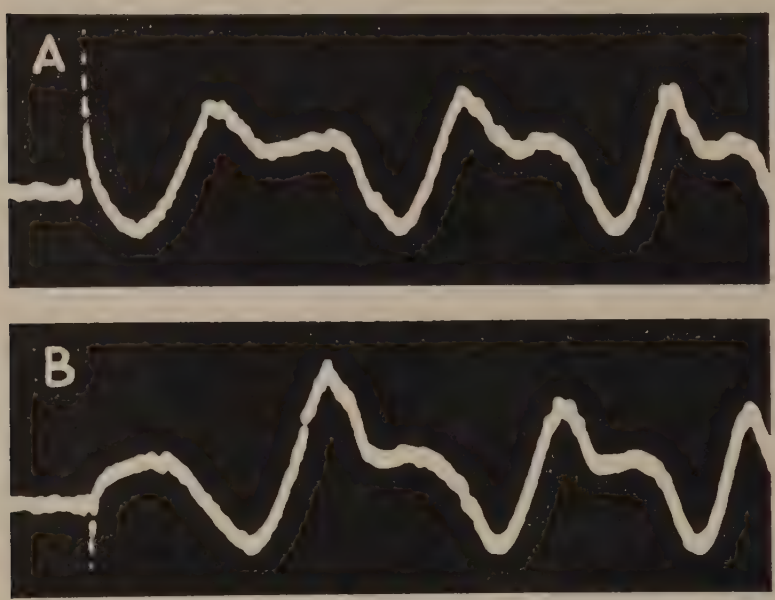

Fig. 13 Influence of the phase of starting on the amplitude of the local response to the first cycle. The records show the effects of the beginning (sharp artifact) of periods of application of a.c. (70 cycles per sec.). In A the current began at the start of the anodal half-cycle; a local response begins to develop at the trough of this half-cycle. In B the current began at the start of the cathodal half-cycle; a striking local response is seen only after the following anodal half-cycle.

J. The influence of cathodal or anodal polarization on the local responses. For these observations electrode *d* was placed at the position of optimum balance. The a.c. was applied through electrodes *a* and *b,* as usual, and through these same electrodes d.c. pulses (20 to 50 msec.), anodal or cathodal with

respect to *a,* were delivered with a frequency that was a submultiple of that of the a.c. The sweep of the cathode-ray oscillograph was synchronized with the pulses. Each of the successive similar sweeps thus showed some a.c. cycles, then several applied simultaneously with the d.c. pulses, and then some later ones.

The changes of the local responses to a.c. during the flow of d.c. and in the postanodal and postcathodal periods are quite similar to those that occur in the local responses to brief d.c. test pulses, anodal or cathodal, in the same circumstances (see Rosenblueth and García Ramos, '51). There is a summation of the local responses to a.c. with those to the long polarizing pulses. On the other hand, the responses to the a.c. decrease when they develop during the course of the positive swings elicited by the pulses.

DISCUSSION

I. The relations between the local responses to a.c. and those to d.c. In the study of Rosenblueth and García Ramos ('51) the conclusion was reached that the local responses to cathodal or anodal d.c. pulses have the same properties, i.e., that they denote the development of the same process. The present data confirm that conclusion and indicate that the responses to a.c. are in turn similar, that they are due to the development of the same process, as follows.

Like the responses to d.c. pulses, those to sinusoidal a.c. decrease or disappear during the refractory period (fig. 4), or when the nerves are reversibly depressed by strong a.c. (fig. 5) or d.c., or by cocaine. The responses to a.c. sum with those to d.c. (section J). All the responses increase according to the same law when the currents are intensified (see fig. 7). They vary in a similar way during or after cathodal or anodal polarization (section J). They have a similar distribution along the nerves (see fig. 12). The responses to a.c., like those to d.c., lead to the initiation of propagated spikes if they attain a sufficient magnitude.

The time course of the responses to a.c. shows no discontinuity at the time that the anodal half-cycle is followed by the cathodal half-cycle. The responses begin shortly after the peak of the inward flow of current; they reach their maximum some time after the beginning of the cathodal half-cycle, and they end after the cathodal peak is past (fig. 10). It is thus clear that the responses to a.c. are the sum of an anodal and a cathodal response, but that the distinction between the two contributions would be arbitrary.

II. The local responses and Lorente de Nó's E_1 potentials. Lorente de Nó ('47) has denied the existence of the local responses. He suggests that an applied current creates an E_1 potential, with a relatively rapid time constant, and that the response of the nerve is a reaction, that is, the establishment of a counter e.m.f., with a slower time constant.

The present results do not support Lorente de Nó's suggestions. The rate of development of the E_1 potential should depend on that of growth or decay of the currents applied, and could thus be made as slow as desired. The rate of the reaction, on the other hand, should depend on the properties of the nerve, and therefore could not be accelerated above the limit imposed by its time constant. It could, however, be slowed at will, by slowing the rate of change of the currents.

Thus, with a.c. of low frequency both the development of the E_1 potentials and that of the reactions would follow the currents, i.e., they would appear as sinusoidal processes with the same frequency as the a.c. Whether or not there be a phase shift, the sum of several sinusoidals of the same frequency yields another similar sinusoidal. Lorente de Nó's hypothesis predicts, therefore, that the records obtained when low frequencies of a.c. are applied should be sinusoidals, but they are not (fig. 6).

The hypothesis proposed by Rosenblueth and García Ramos ('51) is in accord with the present results. If we assume that there is a local response which does not oppose the e.m.f. im-

pressed, and which has an independent time course, this response may be superimposed in the records on the passive sinusoidal component, as is in fact the case.

III. The measurement of the local responses. The method used to measure the responses assumes tacitly that the only significant components superimposed on the passive sinusoidal changes of potential are the local responses. This assumption implies that, unlike what occurs when d.c. pulses are applied, the subsidence of the cathodal and the onset of the anodal half-cycles fail to elicit positive swings of significant amplitude (see Rosenblueth and García Ramos, '51).

In order to test this assumption the following observations were made. The experimental set-up described in section C (see fig. 6) was used. The electrode *d* was slightly off balance. Weak a.c. was applied and a constant fraction of this a.c. was used to supply the sinusoidal that was adjusted to the passive component of the records. The a.c. was then intensified within the subthreshold range. This intensification leads to an equal increase of the amplitude of the sinusoidal base-line and of that of the passive component of the records. If the main or only local active change were the local response the adequate fit of the sinusoidal to the part of the record assumed to denote the passive component should therefore be preserved after the intensification. If, on the other hand, this part of the record includes a positive swing, this swing would increase and the fit would be disturbed.

The results of these observations indicate that the part of the records considered passive includes in fact an active positive swing. The responses to a.c. are thus oscillatory; they consist of an alternation of local responses and positive swings. The similarity between the effects of d.c. and of a.c. on nerve is thus preserved, as was expected *a priori*.

The amplitude of the positive swings, however, was much smaller (less than 5%) than that of the local responses, even in nerves that gave ample positive swings to d.c. pulses. The error made by neglecting the swings in the measurement of

the local responses is therefore small. It may be noted, incidentally, that the equation given in section F results in the development of a slight positive swing (see fig. 11).

IV. The time course of the local responses. The fact that the local responses to a.c. have a duration that is proportional to that of the cycles over a wide range of frequencies (figs. 10 and 11; table 1) is in contrast with the constant duration of the responses to long d.c. cathodal pulses. When these pulses are applied the response begins to develop immediately; it reaches its peak in less than 2 msec.; it then subsides and ends in approximately 10 msec., even though the pulse be much longer With slow-frequency a.c. the response may be much longer. Thus, with 10 cycles per sec. it lasts approximately 0.6 of the cycle, that is, 60 msec. Furthermore, the responses to a.c. are symmetrical, unlike those to d.c. pulses.

These facts suggest that the development and amplitude of the local responses is a function of the rate of the changes of flow of current with respect to time, that is, of the first derivative of the current applied. Thus, with cathodal rectangular pulses the response increases while the flow of current is established, that is, while the axons are being more and more depolarized; it thereafter subsides because the rate of current, and of depolarization, is stable, that is, because the derivative with respect to time is zero. With slow-frequency sinusoidal a.c. the response begins to develop as soon as the axons begin to become depolarized, after the initial anodal polarization; it reaches its maximum approximately when the rate of depolarization is maximal, shortly after the transition from the anodal to the cathodal half-cycles; it then decreases to disappear soon after the axons begin to become repolarized.

It is desirable to emphasize that the phase shift of the responses illustrated in figure 10 denotes a genuine lag with respect to the changes in the impressed e.m.f. If records are taken with electrode *d* 2 to 4 mm away from one of the poles, in the extrapolar region, and the frequency of the a.c. is gradually increased, the sinusoidal, main electrotonic component of the records shows changes of phase with reference to the

records taken from the pole. But the angles involved are quite small when compared with those shown in figure 10. It is clear, therefore, that the phase shifts in the figure are not due to the fact that the records were not taken directly from the pole, but from some neighboring point to the distant crushed end.

The reasons for the lag of the responses are not obvious. We may accept as a fact, however, that whereas with slow frequencies (below 20 per sec.) the responses begin to develop almost immediately after the anodal half-cycle begins to decline, with higher frequencies it only begins to develop later, and with frequencies higher than about 500 per sec. this beginning does not take place until the cathodal half-cycles have been applied for some time. It may be mentioned, however, that a lag of 100° at 700 per sec. is a lag of only 0.4 msec.

V. The stimulation of nerves by alternating currents. The data show that the initiation of impulses by alternating currents is due to the same mechanism that develops when rectangular pulses, cathodal or anodal, are applied (see Rosenblueth and Luco, '50; Rosenblueth and García Ramos, '51). In all instances the impulses arise if, and only if, a sufficiently ample or prolonged local response has developed.

The theory of stimulation by a.c. requires the accurate knowledge of the relations between the intensity of the currents applied and the amplitude of the local responses that develop as a consequence, and also the knowledge of the amplitude or duration of the responses that attain the threshold for spike initiation, and of the conditions, determined by the currents, that modify this threshold. The current theories do not take account of these bases, and are therefore unsatisfactory.

Let us consider as an example Hill's ('36) theory, one of the most complete and mature available. This theory, like others, does not take cognizance of the fact that in stimulation by a.c. not only the cathodal half-cycle, but also the anodal one, is important, since the anodal local response sums with the cathodal. Hill was led to conclude that when high-fre-

quency a.c. begins to flow, the response to the first cycle will be greater if the current begins at the start of the cathodal half-cycle than otherwise; for low frequencies, the effects would be optimal if the current begins at the peak of this cathodal half-cycle. As shown in figure 13 this inference is erroneous; the optimum effects at all frequencies are obtained when the current begins with the anodal half-cycle.

Again, the theory does not take cognizance of the facts that if a local response follows another one at relatively long intervals (0.5 to 10 msec.) the second will be reduced in magnitude, whereas if it follows the first at short intervals (0.1 to 0.4 msec.) it will be greatly increased (Pumphrey, Schmitt and Young, '40; Rosenblueth and Luco, '50; Rosenblueth and García Ramos, '51). In the present experiments the decrease of responses in figure 8 for frequencies greater than about 100 cycles per sec. may be at least partly due to the first effect. And when high frequencies (over 2,000 per sec.) are applied, the fact that spike discharge may not take place until several cycles have elapsed is due to the second effect mentioned.

The hypothesis that spike discharge occurs when the local responses reach an adequate magnitude is compatible with the well known fact that there is an optimum frequency for stimulation by a.c. (see fig. 9, upper curve). At that frequency (about 80 per sec. for the nerves studied here) the amplitude (fig. 9, middle curve) or area (lower curve) of the threshold local responses is small; in addition, the amplitude of the responses elicited by a.c. of a given voltage is high (fig. 8).

The greater intensity needed for stimulation with a.c. of frequencies higher than the optimum is probably due to the decline of the amplitude of the responses illustrated in figure 8. It does not seem necessary to assume that the threshold local responses need to be greater than at the optimum frequency, since although the amplitude of these responses increases their area remains approximately constant (fig. 9).

The existence of an intermediate process, namely the local response, that develops between the application of an electric stimulus and the initiation of a nerve impulse, requires a

redefinition of the term accommodation. The term has been used to designate an increase or a decrease of the threshold due to the passage of currents. It is clear, however, that an increase of the threshold strength or duration of the electric stimuli could be due either to a decrease of the local responses or to an increase of the threshold amplitude of these responses, and that these two possibilities should be recognized and distinguished. Since the local responses have no threshold (Arvanitaki, '39; Rosenblueth and Luco, '50; Rosenblueth and García Ramos, '51), we shall use the term accommodation exclusively to indicate that the threshold amplitude of the local responses that determine spike initiation increases (positive accommodation) or decreases (negative accommodation) as a result of the flow of currents through the axons.

When a.c. is applied with frequencies progressively slower than the optimal, not only do the local responses become gradually smaller in amplitude (fig. 8), but the threshold amplitude is also gradually larger (fig. 9). It is apparent, therefore, that these slowly waxing and waning currents lead to an accommodation, in the restricted sense of the term that we have adopted here. The decline in the amplitude of the local responses to a.c. of constant peak voltage, when the frequency is slowed, is explainable on the basis of the hypothesis that the responses are a function of the derivative of the currents. The maximum value of the derivatives is directly proportional to the frequency.

SUMMARY

In the spinal roots from cats the use of a recording method that largely cancels the passive effects of the currents applied (figs. 1 and 2) allows the ready identification of local responses to subthreshold sinusoidal alternating currents (a.c.) of frequencies ranging from 7 to 800 cycles per sec. (figs. 3 and 6). These responses decrease during the refractory period (fig. 4), and also if the nerves are reversibly damaged by the application of strong a.c. (fig. 5), or d.c., or cocaine.

The changes in the amplitude of the responses as a function of the intensity of the ac. obey a typical law (fig. 7). If the intensity is fixed and the frequency varies, the responses are larger for about 100 cycles per sec., and decrease for lower or higher frequencies (fig. 8).

Much as there is a frequency for which the threshold for spike discharge is lowest (about 80 per sec. in the nerves studied) there is also a frequency at which the amplitude of the just-threshold responses is lowest (fig. 9).

The time course of the local responses to different frequencies of a.c. is approximately constant if the duration of the cycle is chosen as unit of time (fig. 10). This constancy and the fact that different local responses have the same instantaneous amplitudes if the peak amplitude is chosen as unit (table 1, fig. 11) allow the derivation of an equation that describes satisfactorily all the responses (p. 333).

The phase of starting an a.c. train modifies the amplitude of the first local response. This response is largest when a.c. begins with the start of an anodal half-cycle (phase angle of 180°).

The discussion leads to the following conclusions. The local responses to a.c. are similar to those to d.c. pulses; they are the sum of an anodal and a cathodal response and no distinction is possible between the two components (section I). There is no evidence for a reaction of the nerve of the type described by Lorente de Nó ('47, section II). The method used to measure the local responses (fig. 6) is satisfactory, although it neglects the existence of a slight positive swing (section III).

The development and amplitude of the local responses is a function of the first derivative with respect to time of the currents applied (section IV).

The stimulation of nerves by a.c., like that by d.c., depends on the development of a local response of sufficient amplitude or duration (section V). The existence of an optimum frequency is explicable on the basis of the changes of amplitude

of the responses to different frequencies (fig. 8) and of the threshold amplitude at those frequencies (fig. 9). The higher threshold for frequencies lower than the optimum is due to accommodation, defined as an increase of the threshold amplitude of the local responses necessary for spike initiation. The higher threshold for higher frequencies is due to the fact that the local responses become smaller.

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POTASSIUM AND SODIUM EXCHANGES IN RABBIT RED CELLS TREATED WITH N-BUTYL ALCOHOL[1]

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FOUR FIGURES

The problem of potassium and sodium exchange by mammalian erythrocytes has been the subject of a number of investigations (Wilbrandt, '37; Davson, '40, '42; Harris, '41; Danowski, '41; Parpart et al., '47; Ponder, '47, '48; Maizels, '49; Flynn and Maizels, '49). The present paper attempts answers to certain questions about this problem. Is there a "normal" rate of exchange of potassium for sodium? Do potassium and sodium exchange mole for mole? Under the proper diffusion gradient does potassium go back into the cell? Can the rate of exchange be accelerated or slowed and subsequently returned to the "normal" rate? When an equilibrium concentration of potassium and sodium has been attained inside and outside the cell how is the volume of the cell affected? The answer to these questions may permit a partial answer to the broader question of the role of the plasma membrane in the retention of potassium by the mature red cell of certain species of mammals.

Red cells which normally have a high potassium concentration in their interior relative to sodium are the most suitable material for studying potassium retention or loss from red cells. Such cells can be placed in a buffered isotonic NaCl

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environment which does not differ greatly from their normal plasma environment (Parpart et al., '47). This is in contradistinction to the procedure of Davson ('40) in which red cells normally having a high sodium content were exposed to an abnormal and probably injurious environment, namely one rich in potassium.

METHODS

Rabbit blood, drawn by heart puncture, was collected in a flask containing sodium citrate in the amount of 400 mg % (dry). The blood was centrifuged at 3000 G for 10 minutes and the red cells washed three times in a solution containing 9 parts of an isotonic solution of NaCl and one part of a mixture of (isotonic) 0.11 M NaH_2PO_4 and 0.11 M Na_2HPO_4, giving a final pH of 7.09. The effect of washing on the potassium content of the cells was variable and the amount of change was slight in all cases. Throughout the experiments 1 ml of these washed, partly packed cells per 20 ml of experimental solution was used except in the case of those controls in which whole blood was employed. Butyl alcohol was dissolved in the 1% NaCl-phosphate solution and was used in concentrations ranging from 0.25 to 0.40 M. With the higher concentrations no appreciable hemolysis occurred before 30 minutes. All experiments were run at room temperature of approximately 22°C.

In order to study the rate of exchange of sodium and potassium in cells treated with the above concentrations of butyl alcohol it was necessary to devise a means of stopping the action of the alcohol quickly. It was found that rapd packing of the cells by use of a special rotor head for the air turbine centrifuge (Parpart and Ballentine, '43) effectively stopped all action of the butyl alcohol. In figure 1 is shown a cross section of the air turbine head with a side arm leading from a well in the center of the head into the air turbine tube, which has a capacity of 1 ml. This type of head has been designated the Rapid Separator Head (RSH). The cell sus-

pension was injected into the moving turbine by means of a syringe; most of the cells remained at the centrifugal end of the air turbine tube while any excess of supernatant was thrown out of the centrifuge. The cell suspension was injected while the turbine was moving with a force of 17,000 G; the injection time was about 50 seconds. The cells were then completely packed by 10 minutes more centrifugation. The cells in whole blood and other control tubes were packed in the same manner, so that all red cells were accorded the same mechanical as well as dilution treatment. Whole blood con-

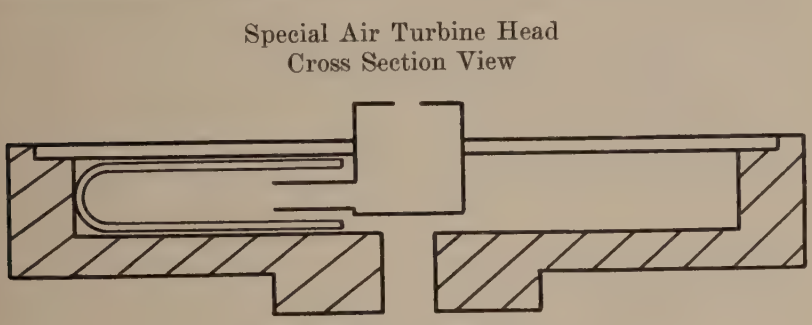

Fig. 1 Cross section view of air turbine rotor head used for rapid separation of cells from supernatant.

trols, packed once in air turbine tubes, and those packed after dilution, in the foregoing manner, did not differ in their potassium contents. It was concluded that the mechanical factor thus introduced did not alter the potassium content.

In the experiments dealing with the slowing of the rate of *K-Na exchange* (fig. 3) the cells were initially exposed to the butyl alcohol for a short time and the action of the alcohol was stopped in the Rapid Separator Head. The cells were then quickly resuspended in 20 ml of 1% NaCl-phosphate solution by emptying the packed cells centrifugally (at ca. 1000 G) into a centrifuge tube containing the known volume of diluent. Subsequently the cells were re-exposed to butyl

alcohol at the desired time and finally packed in the RSH. These packed cells were used for analysis.

The potassium and sodium analyses were made by hemolysing 0.1 ml aliquots of air turbine packed cells in 15 ml of distilled water. The potassium (after an additional one-half dilution) and sodium determinations were made in the standard way using a modified Perkin-Elmer flame photometer.

The hemoglobin concentration of each of the flame photometer dilutions was determined at 540 mμ on a Beckman spectrophotometer. These values for hemoglobin concentration were used as a measure of any volume changes which occurred during the butyl alcohol treatment. Any such volume change of the experimental cells with reference to the control cells was used as a correction factor in calculating the concentration of potassium and sodium.

RESULTS

Figure 2 is a plot of typical results obtained when rabbit red cells are exposed to 0.4 M n-butyl alcohol. It shows that the loss of potassium with time is paralleled by the gain in sodium within the cells and that under the conditions of these experiments the loss of potassium is really an exchange with sodium. When red cells are exposed to 0.35 M concentrations of butyl alcohol the rate of exchange of sodium and potassium is appreciably slower. Table 1 shows the per cent of the original potassium content lost and sodium gained with time resulting from exposure to two concentrations of butyl alcohol. Included in the table is a column showing the volume changes of the cells which occurred with time.

A consideration of such data as are presented in table 1 suggests that the butyl alcohol is in some way altering the cell surface so that potassium and sodium ions can exchange more rapidly than normal. If the principal site of action of the alcohol were the cell surface some insight into the nature of this action could be gained by determining the reversibility of the phenomenon.

The results of an experiment to test the reversibility of the action of butyl alcohol are shown in figure 3. Potassium loss with time only is plotted. The solid dot curve shows loss of potassium from continuous exposure to butyl alcohol. The

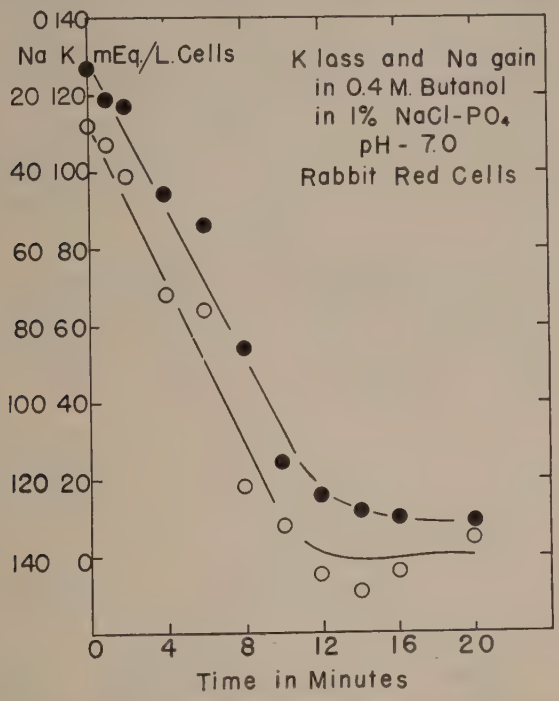

Fig. 2 Plot of potassium loss and sodium gain in rabbit red cells exposed to 0.4 M butyl alcohol. In order to show the equivalence of *K-Na exchange* the sodium ordinate scale has been inverted.

TABLE 1

0.35 M BUTYL ALCOHOL				0.40 M BUTYL ALCOHOL			
Time Min. Exp.	% K lost	% Na gained	% Original volume	Time Min. Exp.	% K lost	% Na gained	% Original volume
0	0	0	100	0	0	0	100
6	10.3	16.9	101	2	7.1	7.8	102
15	31.6	40.7	105	4	24.4	27.5	104
20	47.1	60.8	106	8	51.0	58.6	106
25	52.3	73.6	104	16	91.4	81.4	115
				20	92.2	80.2	119

open circle curve shows potassium loss resulting from an initial exposure for two minutes to butyl alcohol followed by removal of the alcohol for 10 minutes and at this time a re-exposure to the same alcohol concentration. The X curve is the result of the same treatment as the open circle curve except that the period between the first and second exposures to the butyl alcohol was 40 minuts. The curve designated by

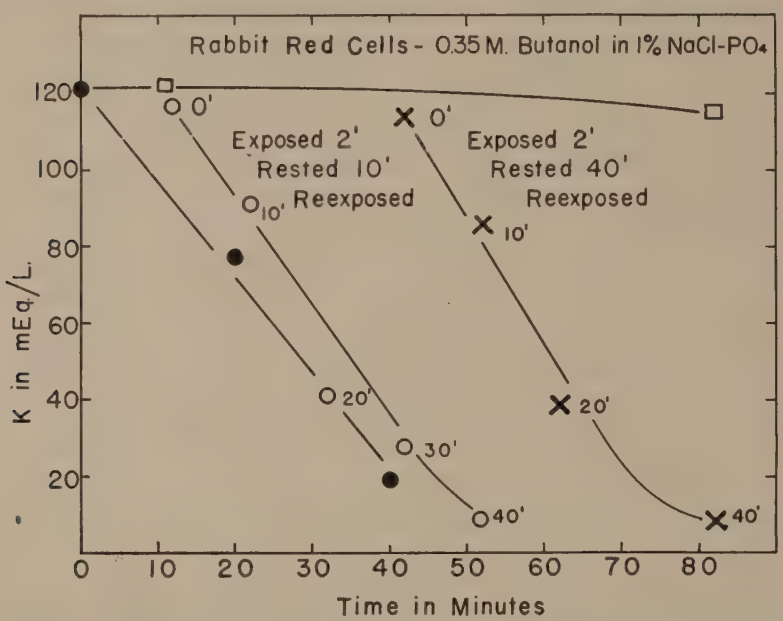

Fig. 3 Plot of rate of potassium loss from rabbit red cells exposed to 0.35 M butyl alcohol. ● = continuous exposure; O = two exposures separated by 10 minute interval; x = two exposures separated by 40 minute interval.

squares, represents potassium loss when cells were exposed at the same dilution to the isotonic NaCl-phosphate solution without butyl alcohol.

Two facts are clearly revealed by figure 3; first that the action of the butyl alcohol is reversible and second that upon the reexposure to the alcohol the rate of loss of potassium is essentially the same as that of a continuous exposure. Both facts support the idea that the action of the butyl alcohol is

confined to producing a reversible alteration of the cell surface, rendering it more permeable as evidenced by the increased rate of loss of potassium ions.

Table 1 contains columns showing that the original volume of the cells increases with time of exposure to butyl alcohol. Figure 4, which includes additional data, shows the volume changes with time for three concentrations of butyl alcohol. These are the results of several experiments. From this graph and from table 1 it seems clear that as the rate of *K-Na ex-*

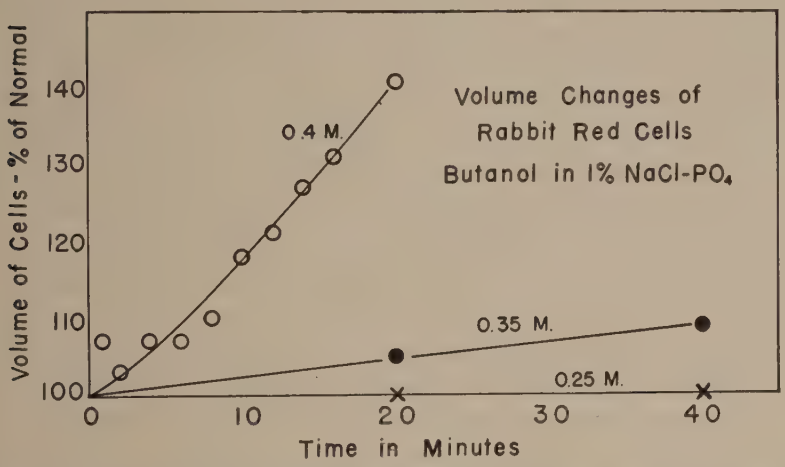

Fig. 4 Plot of volume changes with time occurring in rabbit red cells exposed to 0.25, 0.35 and 0.40 M butyl alcohol.

change is increased the volume of the cells is increased. The significance of this fact and its relation to the colloid osmotic pressure of the cells will be pointed out in the Discussion.

DISCUSSION

There has been considerable confusion of interpretation in the literature on *K-Na exchange* of red cells. This has resulted from the great variety of experimental procedures to which the red cells under observation have been exposed. We intend in the present discussion to limit ourselves to those studies in which the *K-Na exchange* was determined on red

cells of freshly drawn blood, not subjected to any storage period.

The experimental facts reported earlier in this paper demonstrate that sodium ions are exchanged quantitatively for potassium ions in rabbit red cells treated with various lytic concentrations of butyl alcohol. This quantitative exchange precedes by a considerable time the hemolytic action of butyl alcohol.

There is very little data in the literature on the influence of lytic agents on *K-Na exchange*. One of these studies was carried out on human red cells under the influence of low-prolytic concentrations of sodium taurocholate and distearyl lecithin (Ponder, '47a). Under these conditions Ponder found from one and eight-tenths to two and six-tenths times more sodium entering than potassium leaving the cell. However, the time course of exchange was slow under his experimental conditions, so that only from 6 to 15% of the cell potassium was lost. With respect to potassium, Ponder believed the loss was approaching a new steady state far from diffusion equilibrium. It would seem that any conclusion relative to potassium loss and sodium gain should be based on a time-course of exchange which is followed at least a large part if not all the way to equilibrium. Indeed, in a subsequent paper, the time course of potassium loss at 4°C. from human red cells under the influence of n-butyl alcohol and resorcinol the data parallel our results on potassium loss (Ponder, '48a). Unfortunately, no sodium data are given so that it is not possible to make this comparison. Our own results on sodium would lead us to suspect, however, that a similar quantitative exchange took place.

It is quite possible that the approximate quantitative exchange observed by us is not followed by all lysins and different types of red cells; however, we found the same effect with ethyl alcohol and with dodecyl sulfate in rabbit red cells. Recently Sheppard and Beyl ('51) reported a quantitative *K-Na exchange* in human red cells exposed to x-rays during a time interval of 26 hours.

Only one author appears to have considered the rate of potassium loss at 25°C. of the red cells of freshly drawn blood when exposed to isotonic saline solution (Ponder, '49). His experiments on human red cells showed that the potassium of the interior of the cell proceeds towards diffusion equilibrium with its environment under these conditions. Again no data on sodium are available.

The present paper offers no information as to a "normal" rate of *K-Na exchange* in rabbit red cells in vitro. Under the experimental conditions reported here, the washed, untreated controls did not exhibit any *K-Na exchange* within the experimental error of measurement. However, the rapid rate of exchange obtained in our experiments with 0.4 M butyl alcohol in isotonic NaCl-PO_4, namely, diffusion equilibrium attained in 20 minutes, is in marked contrast to the experiments of Parpart et al. ('47). These workers showed that under well controlled conditions the attainment of diffusion equilibrium for potassium from human red cells may require as much as 40 days. Thus it would appear that it is possible to create conditions so that the normal permeability of red cells to cations such as potassium and sodium is very slight. It would seem that the term permeability to ions such as potassium and sodium should always be defined with reference to the conditions under which it is determined. This perhaps obvious fact has frequently been neglected.

No reports have been found in the literature which described experiments in which the rapid *K-Na exchange* is checked and then subsequently begun again. Davson ('39) in his studies with glucose as a suspension medium presents data "demonstrating the reversibility of the induced permeability to potassium." Reversibility, as Davson appears to use the term, means a decrease in the rate at which potassium is lost from the cells; for after the addition of NaCl to the glucose medium, he found the cells continue to lose some potassium although not as rapidly as in glucose alone. We are using the term reversibility to apply to the situation where an increase in the rate of loss of potassium induced by the ad-

dition of a particular agent to the environment of the cell is brought back to the rate of loss obtained in the corresponding control solution to which the agent had not been added.

The experiment plotted in figure 3 shows that the rate of *K-Na exchange* can be accelerated and slowed and, in fact, returned to a rate scarcely exceeding that of the control. This indicates that the action of the butyl alcohol in accelerating the *K-Na exchange* is reversible. The rapidity with which its action can be reversed by removing the cells from the butyl alcohol containing environment supports the idea that the site of action of the butyl alcohol is the cell surface and not the interior of the cell. Butyl alcohol may be acting by altering the lipo-protein complex in plasma membrane (Parpart and Ballentine, '51). If so it is doing this in a reversible manner.

If, as some investigators suggest (Dean, '41; Harris, '41; Danowski, '41; Ponder, '47; Maizels, '51), the retention of potassium in cells is by some metabolic means, e.g., a pump, then interference with the metabolism of the cell by the butyl alcohol should require some time for recovery and re-exposure to butyl alcohol should result in a more rapid *K-Na exchange* than that obtained initially. Assuming reasonable times for metabolic recovery to take place our experimental result is not in agreement with the metabolic idea. There is an additional reason for believing that metabolic processes are playing a negligible role in these experiments. It has been suggested by Harris ('41) that glucose energy is in some way involved in preventing potassium loss from red cells. Recent work by the authors ('50) indicates that careful washing of red cells removes 80 to 85% of all reducing material, e.g., glucose, originally associated with cells and plasma. It is thought likely that all diffusible sources of glucose energy are removed from red cells by such washing; what proportion remains is uncertain. The cells used in these experiments were washed cells and as such were energy-poor and would be expected to recover metabolically in a slow rather than a rapid fashion.

Therefore, the most likely interpretation of the experimental results in figure 3 is that the butyl alcohol has a reversible effect on the membrane alone, which permits the more rapid *K-Na exchange;* removal of the butyl alcohol reverses this effect. This experiment gives no answer as to the nature of the action of the alcohol on the cell surface. The above description does, however, rather sharply limit the idea of recovery and to a large degree precludes any role for metabolic energy in this case.

As shown in table 1 and figure 4, the volume of the red cells in the higher (0.4 and 0.35 M) concentrations of alcohols increases in a regular manner with respect to length of exposure. At the lower (0.25 M) concentration, however, no volume change was observed, though diffusion equilibrium of potassium and sodium was attained. These observations confirm the volume changes implied from the hemolysis studies of Jacobs and Willis ('47). They also show that the failure of Ponder ('48) to observe volume increases of cells exposed to butyl alcohol was due to the low concentrations, 0.086 and 0.172 M, which he used. Therefore, Ponder's ('48) data were not sufficient to permit him to negate the interpretation Jacobs and Willis ('47) placed on their data.

The normal volume of a red cell is the resultant of a balance between the tendency of the colloid osmotic pressure to move water into the cell and the low permeability to cations which will tend to move out of the cell any water that has entered under the influence of the colloid osmotic pressure. The limiting factor, no hemolysis occurring, in the effect of colloid osmotic pressure on the volume of a cell, is thus the opposition to such volume change on the part of slowly penetrating cations. When, however, cation permeability increases to the point where it is approaching that of water, the cell will increase in volume, since the colloid osmotic pressure is no longer sufficiently opposed. The rate of this volume change will be related to the relative rates of water and of cation penetration and completely independent of a Donnan effect. Thus, even at equilibrium exchange of potassium for sodium no vol-

ume increase will occur unless the rate of passage of potassium and sodium across the membrane is sufficiently rapid. In addition only those cations present in relatively large percentage of the total will have appreciable influence on the volume of the cell.

It can be concluded, therefore, that at higher concentrations of butyl alcohol the rate of penetration of potassium and sodium across the membrane in both directions became sufficiently rapid to permit the colloidal osmotic pressure of hemoglobin to cause the measured volume increase of the cell. On the other hand, at the low concentration of butyl alcohol this penetration rate of potassium and sodium was sufficiently low so that the colloidal osmotic effect could be balanced by the relative, to water, impermeability of the cations.

SUMMARY

1. Rabbit red cells exposed to butyl alcohol, ethyl alcohol or dodecyl sulfate for time periods shorter than those necessary to initiate hemolysis, were found to lose potassium and gain sodium at the same rate. An essentially equivalent exchange of these ions was found in this study.

2. The action of the butyl alcohol, as determined by variations in the rate of *K-Na exchange,* could be reversed with a consequent reduction of the rate of exchange to about that of levels shown by untreated control cells. Reexposure to the alcohol induced a rate of exchange equal to that produced by the initial exposure.

3. It was suggested that the primary action of the butyl alcohol was in altering the cell surface in such a manner as to render it more permeable to cations. A disorientation of such surface components as the lipo-protein complexes would account for the results obtained. This disorientation is reversible at low concentrations and partly reversible at higher concentrations of butyl alcohol.

4. The cells exposed to butyl alcohol increased in volume relative to unexposed controls. The rate of this increase was

correlated with increased cation permeability as shown by increase in the rate of *K-Na exchange*. It was suggested that the volume increase occurred as a consequence of an increase in effectiveness of the colloid osmotic pressure of the cells owing to the increase in cation permeability.

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THE RELATIONSHIP OF AUXIN TO ELECTRICALLY INDUCED GROWTH RESPONSES IN THE AVENA COLEOPTILE[1]

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SIX FIGURES

INTRODUCTION

In recent years it has been demonstrated that application of direct current of the order of 10 to 30 μ amperes to the *Avena* coleoptile causes it to bend. Such current may be applied either transversely (Schrank, '48a) or longitudinally to the apical regions (Kögl, '33; duBuy and Olson, '38; Schrank, '49). Recently assembled evidence indicates that these electrically induced curvature responses are due to growth, which apparently is dependent on auxin.

One series of experiments has shown that phototropic bending toward 200-meter-candle-seconds of unilateral illumination can be influenced by transversely applied direct current (Schrank, '48b). More specifically these results indicate that both electrically and light induced bending are restricted by a common limiting factor, and that the effects of light and current are to some extent additive. The implication is that the applied current and illumination both require the same curvature controlling mechanism, which presumably is some form of auxin.

Additional observations, which support this idea, have been published. Preliminary marking experiments (Schrank, '48a) have shown that both sides of the coleoptile continue to elongate after current is transversely applied. The side on which

[1] Supported by The Research Institute of The University of Texas.

the negative contact of the current applying circuit was placed lengthens more rapidly than the opposite side, which results in bending in the indicated direction. These facts, as well as other considerations (Schrank, '48a, '48b), show that electrically induced curvatures are growth phenomena.

Thimann ('34) has shown that there is a gradient of auxin distribution along the longitudinal axis of the *Avena* coleoptile. The highest concentration appears in the apical 2 mm, while the lowest concentration is found in the most basal cells. If a given current (10 μ amperes for 10 minutes) is applied at various levels below the apex of the coleoptile, the magnitude of the induced curvature decreases as the current applying contacts are moved further below the apex (Schrank, '48a). Thus the electrically induced bending seems to be dependent on the quantity of available auxin.

The experiments herein reported were performed to definitely establish whether or not the curvature of the *Avena* coleoptile resulting from applied direct current is dependent on auxin requiring processes.

EXPERIMENTAL CONDITIONS

All of the experiments were performed in an air-conditioned dark room with the temperature regulated at 25.5 ($\pm$ 1)°C. During an individual experiment the maximum variation was never more than $\pm$ 0.2°C. Illumination was obtained from neon gas in ruby glass tubing which emits only wave lengths longer than 6074 Å. Control experiments have shown that the *Avena* coleoptile is not sensitive to this light.

The electrical measurements were made with a duBridge amplifier ('33). Tapered glass tubes 1 mm in diameter were used as contacts with Shive's solution made with distilled water as the contact medium. Two pairs of isoelectric ($\pm$ 0.5 mv) zinc-zinc sulphate electrodes were employed. One pair was used for the application of current and the other pair for measurement of the electrical responses. Seedlings of a pure strain of *Avena sativa* (U. S. Department of Agriculture,

C. I. 2020, Victory Strain) [2] were used in all experiments. The seeds were husked, soaked, sprouted and transplanted to individual holders in the usual manner. The plants were kept in the dark except for the sprouting period, during which they were illuminated with red light (60 watt General Electric, dark ruby photographic bulb) to inhibit the growth of the first internode. Only straight, intact coleoptiles 30 ($\pm$ 1) mm in length, with a well developed root system and with the primary leaves extended into the tip of the sheath, were used in these experiments.

An India ink spot was placed on the coleoptile 3 mm beneath the apex. Then the seedling was set up in the apparatus and allowed to remain undisturbed for a period of at least 30 minutes before starting the experiments. The ink spot was followed on the horizontal ocular scale as one measurement of curvature. A wet razor blade was used to cut off approximately 1.5 mm of the apex.

EXPERIMENTAL RESULTS

Current applied transversely

Electrical responses. In these experiments the contacts were placed on opposite sides of the coleoptile 7 mm below the apex. Ten μ amperes of direct current were applied for two minutes in this series. During the first 10 minutes the inherent electrical polarities were measured. Current was applied between the 11th and 13th minute, and the next electrical polarity measurement was taken immediately after the 13th minute. Typical electrical responses are presented in figure 1.

Curve I (open circles with diagonal lines) shows the average electrical responses of the control plants, which were decapitated only at the end of the experiments so that measurements of angular curvature comparable to the rest of the plants could be made. (All curves in this paper are averages of 6 or more experiments.) Curve I is very similar to re-

[2] Generously supplied by the U. S. Department of Agriculture.

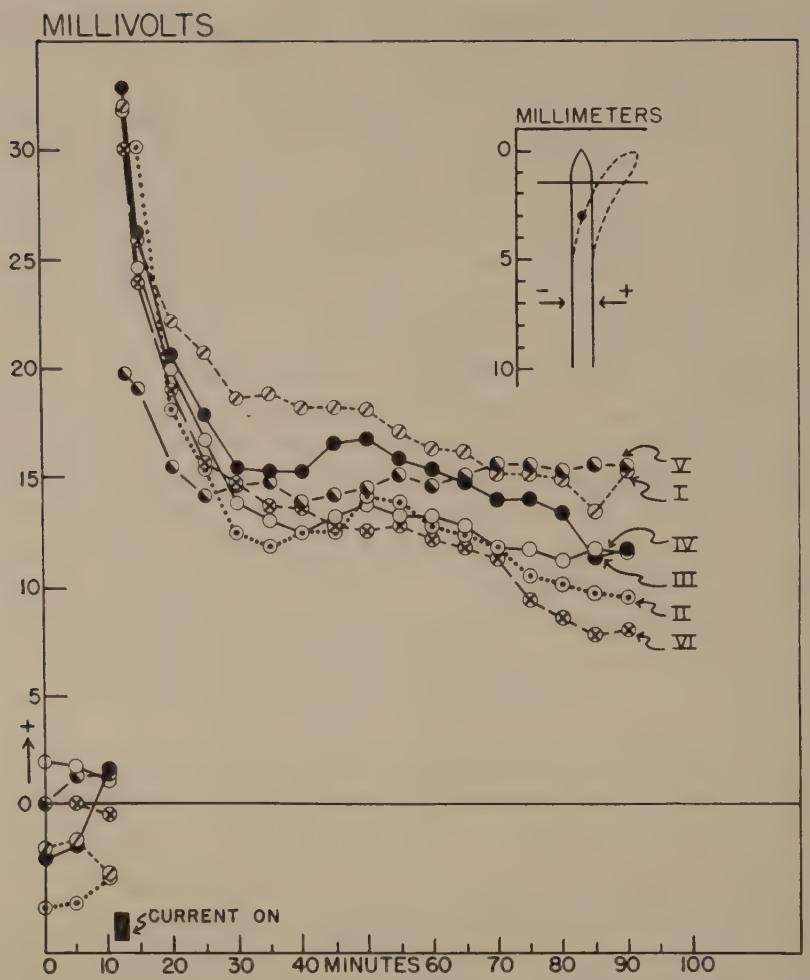

Fig. 1 Electrical responses of Avena coleoptiles to 10 microamperes of direct current applied transversely for two minutes at a level 7 mm below the apex. Each curve is an average of 6 or more experiments. Current was applied at the indicated times:

Curve I, 79 minutes before decapitation.
Curve II, 1 minute after decapitation.
Curve III, 30 minutes after decapitation.
Curve IV, 60 minutes after decapitation.
Curve V, 103 minutes after decapitation.
Curve VI, 120 minutes after decapitation.

sults previously obtained when 10 μ amperes were applied transversely for 10 minutes (Schrank, '48a). Since the current was applied for only two minutes in the present experiments, the initial electrical polarity that is established is appreciably smaller than previously reported.

The results shown by curve II were obtained when current was applied one minute after decapitation. This curve may be treated as a control to indicate the effects of the actual process of decapitation. For curves III (solid circles), IV (open circles), V, and VI the current was applied 30, 60, 103 and 120 minutes after decapitation. (The curves in figures 1 through 4 are numbered to be consistent with this convention. Curve number II always indicates that the current was applied one minute after decapitation, etc.) With one possible exception, the minor differences between the curves in figure 1 are not considered significant. It is noted that the maximum electrical polarity indicated by curve V is only 20 mv, which is slightly more than half as large as shown by the remainder of the curves. The importance of this difference is not apparent at the present time.

The results in figure 1 permit the general conclusion that the transverse electrical polarity established by applied current is independent of the time after decapitation when the current was applied. This also means that the electrical polarity, established in this way, does not require the presence of the apical cells.

Curvature responses. Figure 2 presents the average curvature responses of the same coleoptiles whose electrical changes have just been considered. The graphs represent the rate of horizontal movement of the India ink spot toward the electropositive contact of the current applying circuit. (See inset in the figure.) Averages of the final angular curvatures, measured by the Judkins ('45) method, are inserted at the end of each corresponding curve.

Curves I and II in this figure are practically identical. The coleoptiles used in the experiments for curve I were decapitated at the end of the experiment and those for curve II were

decapitated one minute before the current was applied. Seedlings which had the source of auxin (the apical cells) intact during the course of the bending develop a slightly greater

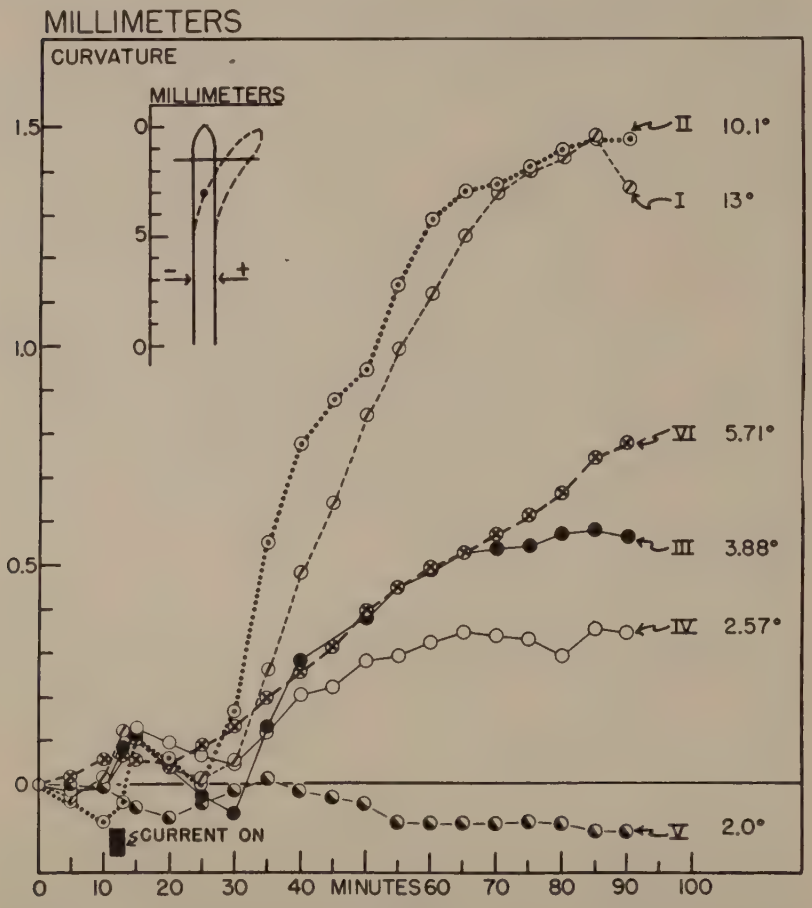

Fig. 2 Curvature responses of Avena coleoptiles to 10 microamperes of direct current applied transversely for two minutes at a level of 7 mm below the apex. Each curve is an average of 6 or more experiments. Current was applied at the indicated times:

Curve I, 79 minutes before decapitation.
Curve II, 1 minute after decapitation.
Curve III, 30 minutes after decapitation.
Curve IV, 60 minutes after decapitation.
Curve V, 103 minutes after decapitation.
Curve VI, 120 minutes after decapitation.

angular curvature. The discrepancy between the curves and the corresponding final angular curvature is due to differences in bending which are apical to the India ink spot.

Comparison of curves II, III, IV and V and their final angular curvatures makes it evident that bending decreases as the interval between decapitation and current application is increased. The minimum curvature, as represented by curve V and 2.0 degrees, was observed when the current was applied 103 minutes after decapitation. This minimum curvature is toward the positive contact even though the point marked by the India ink spot has moved in the opposite direction. The angular curvature measured in these experiments is in and above the contact region. Previous work has shown that there is a secondary curvature basal to the contacts which is toward the negative pole of the current applying circuit (Schrank, '48a). This basal curvature is small, but since it is further away from the apex, it accounts for the reversed horizontal tip movement. When the current was applied 120 minutes after decapitation (curve VI) an average curvature of 5.71° was observed. This indicates a recovery of the ability to bend.

Since it is rather generally accepted that the auxin content of the coleoptile stem reaches the minimum about 100 minutes after decapitation (Went and Thimann, '37; Went, '42), it appears from the data in figure 2 that bending, induced by transversely applied direct current, is dependent on the quantity of auxin that is present.

Current applied longitudinally

Electrical responses. In these experiments the contacts were placed on the same side of the coleoptile 4 and 9 mm below the apex as is indicated in the inset of figure 3. Thirty μ amperes were applied for two minutes in this series, with the basal contact always connected to the positive pole of the current applying circuit so that the direction of current flow would be a constant factor. The curves are numbered as previously explained.

As was the case when the current was applied transversely, the longitudinal electrical response curves in figure 3 are remarkably similar. They are also consistent with previous results (Schrank, '49). Therefore it is apparent that the electrical changes of the coleoptile resulting from longitudinally applied current are also independent of the presence of the auxin producing apical cells.

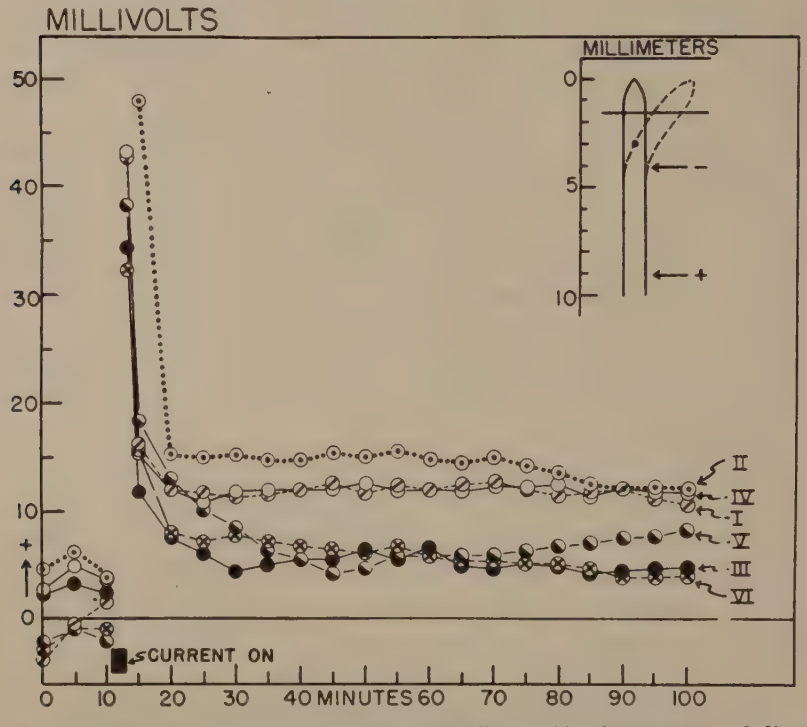

Fig. 3 Electrical responses of Avena coleoptiles to 30 microamperes of direct current applied longitudinally for two minutes. Contacts were placed on the same side of the coleoptile, 4 and 9 mm below the apex. Each curve is an average of 6 or more experiments. Current was applied at the indicated times:

Curve I, 79 minutes before decapitation.
Curve II, 1 minute after decapitation.
Curve III, 30 minutes after decapitation.
Curve IV, 60 minutes after decapitation.
Curve V, 103 minutes after decapitation.
Curve VI, 120 minutes after decapitation.

Curvature responses. When the current (30 μ amperes for two minutes) is applied longitudinally, as indicated in the inset of figure 4, the resultant bending is always toward the side on which the contacts were placed. This confirms previous observations by duBuy and Olson ('38) and Schrank ('49). The rates and the final magnitudes of curvature are also consistent with previous results (Schrank, '49). All of the curves in figure 4 show the highest rate of bending during the first 5 minutes after current application. This fact was not revealed in figure 2, when the current was applied transversely, but it is consistent with previous results when current was applied longitudinally (Schrank, '49). This unique point deserves further experimental analysis.

It is at once apparent that the curves in this figure fall in the same general sequence as those in figure 2. As before, there is a decrease in the rate and magnitude of curvature as the interval between decapitation and current application is increased. Curve IV (current applied 60 minutes after decapitation) represents the minimum curvature in this group, which means that the minimum for this series is reached somewhat earlier than in the previous instance. When the current was applied 103 and 120 minutes after decapitation, recovery of curvature was observed (curves V and VI).

The results in figure 4 are also interpreted to mean that curvatures induced by longitudinally applied direct current are dependent on auxin requiring processes.

Elongation experiments

Figure 5 shows the average rate of elongation of 7 intact coleoptiles. The curve has not been corrected for the inherent decrease in rate of elongation that is characteristic for coleoptiles of this age. After the first 30 minutes, the apical 1.5 mm of each plant were removed as in the previous experiments. The short solid lines parallel to the curve, which are extended from the solid circles, represent the slope of the average curve at the point indicated. Numerical values of the slopes, obtained by graphic methods, are included.

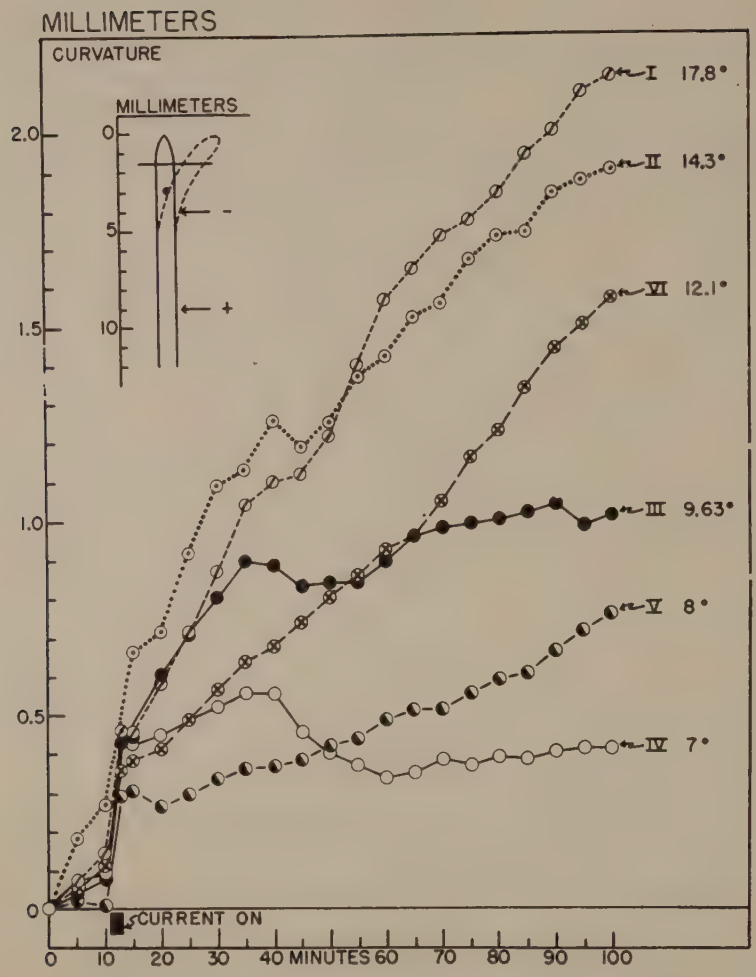

Fig. 4 Curvature responses of Avena coleoptiles to 30 microamperes of direct current applied longitudinally for two minutes. Contacts were placed on the same side of the coleoptile, 4 and 9 mm below the apex. Each curve is an average of 6 or more experiments. Current was applied at the indicated times:

Curve I, 79 minutes before decapitation.
Curve II, 1 minute after decapitation.
Curve III, 30 minutes after decapitation.
Curve IV, 60 minutes after decapitation.
Curve V, 103 minutes after decapitation.
Curve VI, 120 minutes after decapitation.

This curve represents a duplication of the classically accepted observation of the effect of decapitation on the rate of elongation (Dolk, '36; Went, '42). These measurements were repeated in this investigation to have a record of this phenomenon for the same material and under the same con-

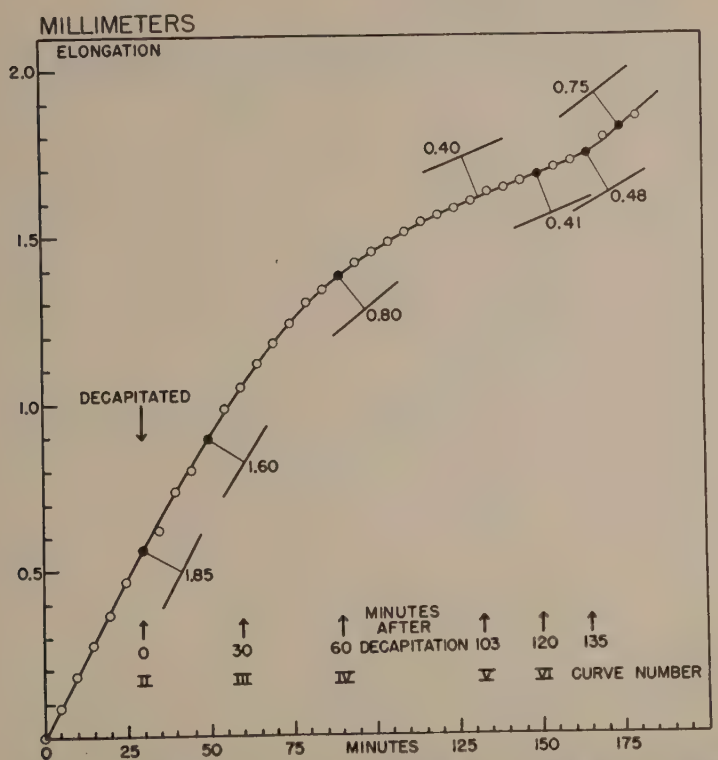

Fig. 5 Average elongation of 7 coleoptiles before and after decapitation. Solid lines parallel to the curve projected from the solid circles of the curve indicate the slope of the curve at that point.

ditions that were used in the current applying experiments. As would be expected from the original experiments, the rate of elongation decreases after decapitation until the minimum rate is observed 120 minutes later. This minimum is followed by a gradual increase in rate, which is due to the functional regeneration of the apical cells.

DISCUSSION

To abridge the observations made in this investigation and to facilitate relevant comparisons, the graphs in figure 6 were prepared. Curve I in this figure was obtained from the

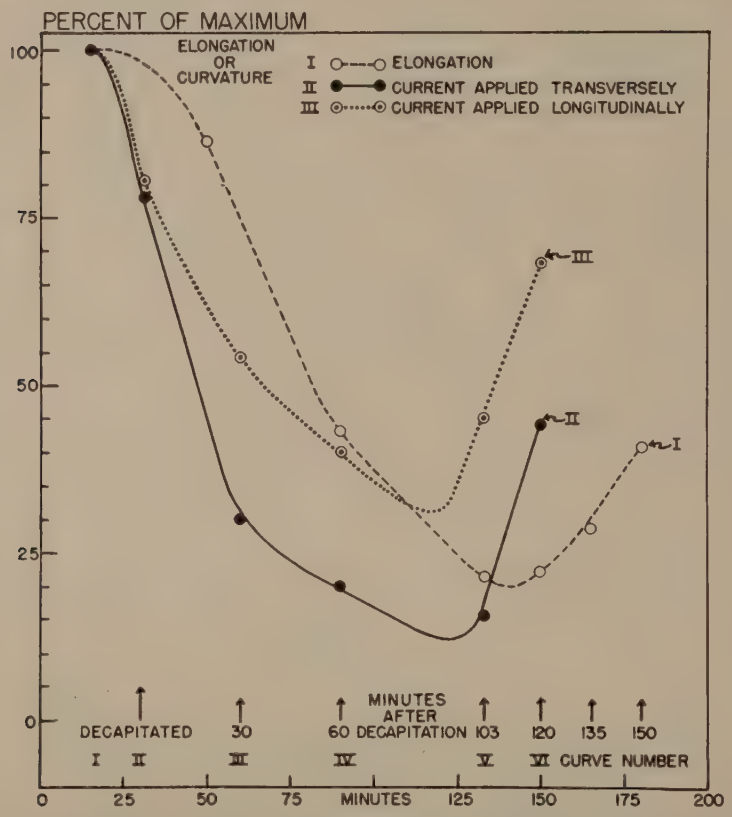

Fig. 6 Comparison of rates of elongation on a percentage basis to curvatures of Avena coleoptiles induced by direct current applied at various times after decapitation. Methods used for preparing the curves are described in the text.

elongation data in figure 5. The maximum rate of average elongation, which is represented by a slope of 1.85 in the curve of figure 5, was taken as 100%. Then the remainder of the slopes, at the indicated times after decapitation, were calculated as fractions of 1.85. These percentages are plotted as

curve I in figure 6. The various times after decapitation at which the current was applied are indicated on the horizontal axis. Curve I indicates that the minimum elongation rate, which is about 20% of the maximum, is reached between 103 and 120 minutes after the apex was removed. Thirty minutes later the elongation rate has increased to 40.5% of the maximum.

Curve II, from experiments in which the current was applied transversely, was obtained from figure 2. In this instance 13° represents the maximum or 100% bending. All of the rest of the average angular curvatures are expressed as fractions of 13°. It appears from curve II that the least curvature would be attained if the current were applied about 95 minutes after decapitation. When curve II is compared to curve I, it becomes apparent that curvature due to transversely applied current goes through the same cycle of events as elongation after decapitation. The curvature responses precede the elongation changes by 20 to 30 minutes. This could be a reflection of the fact that current as applied in these experiments affects only a limited apical region, while the entire length of the coleoptile contributes to the measured elongation. Apparently this means that the effective auxin disappears from the apical cells before it does from the basal section, and that the more apical cells are the first to receive the newly produced auxin after the functional apex reappears. These basal cells are evidently sustaining the elongation before the minimum is reached, while the apical cells are the first to start lengthening again after the slowest rate had been attained.

Went ('42) performed a series of experiments to determine the effect of geotropic stimulation applied to the *Avena* coleoptile at varying times after the apical 5 mm had been removed. He found that geotropic sensitivity rapidly disappeared during the first 30 minutes after decapitation. These facts, along with other observations, led Went to maintain that the diffusible auxin in the coleoptile is correlated with tropisms, while the growth rate is more likely related to the

extractable auxin. Curve II in figure 6 is practically identical with Went's geotropic sensitivity curve. This permits the suggestion that the curvature resulting from transversely applied direct current might also be dependent on the free moving fraction of auxin.

Curve III in figure 6 was derived from figure 4. In this instance 17.8° is the maximum or 100%, which was used as the basis to express the remainder of the average curvatures. The sequence of events represented by this curve is essentially the same as in the two previous ones. Interpolation of curve III indicates that the minimum curvature due to longitudinally applied current would be observed if the current were applied 90 minutes after decapitation. Again the bending changes precede the elongation changes, the probable reasons for which have been elaborated in the previous paragraphs.

The significant features in figure 6 were checked by preparing a second set of similar curves. In this instance the rates of elongation at various times after decapitation were compared to the electrically induced curvature at the corresponding times as measured by the slopes obtained from the initial and final points of the curves in figures 2 and 4. These curves are omitted because they present essentially the same facts as figure 6. Only two minor differences were noted. First, curve II and III of the omitted figure are parallel for the first 110 minutes of the experiment. Secondly, the recovery of curvature after the minimum is slightly larger when measured in this way. At 120 minutes after decapitation this method (taking the slope of the initial and final points of the curves representing horizontal tip movement) indicates 47.5% of the maximum curvature for transversely applied current as compared to 44% when the angular curvature is taken as the criterion of bending. For longitudinally applied current the corresponding difference is 79% as compared to 68%.

Experimental evidence, which was cited in the Introduction, definitely indicates that curvature responses of *Avena* coleop-

tile to direct current applied to apical regions are dependent on auxin requiring processes. If the classical statement of "Ohne Wuchstoffe kein Wachstum" (Dolk, '36) is correct, and there is much evidence which indicates that it is (Went and Thimann, '37), then the data herein presented permit the following definite conclusion. Bending responses of the coleoptile caused by application of direct current are auxin requiring growth responses. These observations also raise the question of whether electrically induced curvatures are due to a redistribution of auxin. The previously discussed indication that the free moving fraction of auxin might be involved, emphasizes the need for additional experimentation on this aspect of the problem.

SUMMARY

1. Experiments are described in which direct current was applied to the *Avena* coleoptile before removal of the apical 1.5 mm and at specified intervals after decapitation. Both the electrical and curvature responses were measured.

2. Ten microamperes of direct current applied transversely for two minutes at a level 7 mm below the apex establishes a transverse electrical polarity and causes the coleoptile to bend toward the positive pole of the current applying circuit. Removal of the apex had no effect on the electrical polarities that were established. The time after decapitation at which the current is applied determines the amount of bending. Minimum curvature was observed when the current was applied 103 minutes after decapitation.

3. Thirty microamperes were applied longitudinally for two minutes with both contacts placed on the same side of the coleoptile 4 and 9 mm below the apex. The electrical polarities established are independent of the presence of the apex. Bending toward the contact side depends on the length of the interval between decapitation and current application. Minimum curvature was observed when the current was applied 60 minutes after decapitation.

4. The results prove that current induced electrical responses of the *Avena* coleoptile are independent of the auxin mechanism that is required for curvature.

5. Comparison of the curvatures to rates of elongation at corresponding times after decapitation reveals that the bending responses to current, applied either transversely or longitudinally, are dependent on auxin requiring growth processes..

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SULFHYDRYL DETECTION BY HISTOCHEMOGRAPHY

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THREE FIGURES

INTRODUCTION

Experiments performed in 1948 and 1949 (Boyd and Board) indicated that animal tissues were capable of forming images on the photographic plate by direct contact. Smears of bone marrow, and sections of spleen, kidney, liver, heart and lung of normal rats were found to produce such images. It was felt that these images were caused by chemical reaction of tissue substances with the silver bromide grains of the emulsion. The studies reported in this paper represent an attempt to determine the more specific nature of the substances in the tissues responsible for the phenomenon.

MATERIALS AND METHODS

Eastman NTB plates (emulsion batch no. 415633, 25 μ thick, on 1 × 3 inch plates) were used for all experiments. This emulsion was not affected by hydrogen peroxide (Keenan). Guinea pigs were the source of fresh tissues. Chemographs were produced in the following manner: after the animals were sacrificed with ether, the organs and tissues to be studied were removed and taken to the dark room; under a series I Wratten Safe Light (10 watt bulb, distance greater than 40 cm) 2 to 3 mm thick slices were made with a razor blade

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and placed immediately on the emulsion with the aid of forceps; light pressure of the forceps was used to insure contact of all parts of the slice; the slides were placed in a black plastic box, sealed from light and stored at 25°C. during the exposure period (usually 4 hours); the tissues were then carefully removed with forceps, after which the slides were soaked in tap water for 4 minutes before the emulsion surface was gently washed free of tissue debris with cotton sponges and water. The plates were developed for 4 minutes in full-strength Kodak D19, fixed 20 minutes in Kodak Acid-Fixing Bath, and thoroughly washed.

In the handling of homogenates, extracts, and pure chemicals, sponges consisting of cotton batting rolled into balls about 0.5 cm in diameter were dipped into the substance to be tested, placed on absorbent paper, flattened out with forceps, and then placed on the emulsion. In all other respects the sponges were treated like organ and tissue slices.

It was felt that the adoption of these simple methods was justified by experiments which demonstrated that the thickness of the section or the pressure upon it might be varied within wide limits without causing important changes in the photographic densities of the images as measured with densitometer. For instance, densitometer measurements of images produced by liver slices 1 mm and 10 mm thick, by liver and kidney slices with and without external pressure applied (43 gm/cm), and by a solution of glutathione applied with cotton sponges of varied size, volume, and compactness clearly indicated that neither volume nor pressure had an appreciable effect upon the image produced.

RESULTS

1. Organ and tissue slices. A series of exposures showed that guinea pig liver, kidney, spleen, heart, lung, gut, pancreas, brain, eye, striated muscle, bone marrow, testis, ovary, and thyroid were able to produce images on the NTB plate, but that subcutaneous fat failed to do so.

The graph in figure 1 represents the density of image of guinea pig liver slices plotted against hours of exposure to the NTB plate. Each point represents the average density of 6 liver images, for each hour, commencing with a one minute exposure. A sharp rise is noted during the first two hours, after which a gradual increase to 6 hours is seen. At this point

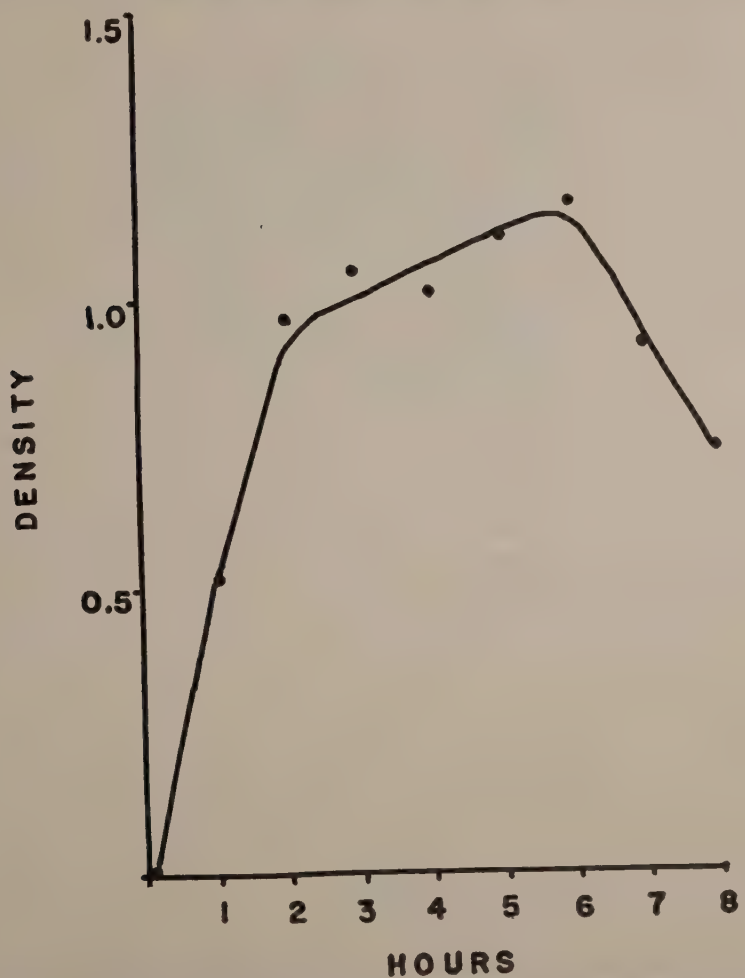

Fig. 1 Image densities of liver slices plotted against exposure time. Each point represents the average density of images produced by 6 different liver slices at each hour of exposure.

the densities decrease. This curve is considered to be typical of the development of images of the guinea pig liver and was used as a guide to exposure times for other tissues (heart, kidney, muscle, lung and spleen) which also required from 2 to 6 hours' exposure to produce mature images. Table 1 gives the average densities of the 4-hour exposures of 6 slices of liver, heart, kidney, lung, striated muscle, spleen and fat.

An examination of the liver images for characteristics other than density shows a progressively widening border with increasing time of exposure; the border is pale-grey in shade and about 1 mm in width, shown in figure 2. The liver image is composed of indistinctly outlined, lobule-like areas and the

TABLE 1

Image densities of guinea pig organ slices[1]

LIVER	HEART	KIDNEY	LUNG	MUSCLE	SPLEEN	FAT
1.01	0.75	0.46	0.39	0.07	0.04	0

[1] Each figure represents the average density of 6 guinea pig slices.

main body of the image is grey-black in appearance. Although not apparent in the photomicrograph, there was usually a fine silver mirror about the periphery of the image.

Sections of liver were baked for one hour at 60°C. or boiled in ethyl alcohol for one minute, to inactivate thermo-labile substances. In each case the capacity for image-formation remained. By using a number of enzyme inhibitors, it was found possible to narrow the approach to the problem. Iodoacetic acid, mercuric chloride and copper sulfate depressed the formation of the images when slices were dipped in a 10^{-2} molar solution of these substances. However, it was shown that mercury compounds ($HgCl_2$ and p-chloromercuribenzoic acid) and copper compounds desensitized the plate to such an extent that it was not certain whether the inhibition of the tissue was real or apparent. Iodoacetic acid (10^{-2} and 10^{-3} molar) was satisfactory, for it did not desensitize the plate, but did inhibit formation of the image.

2. Homogenates and extracts. It was also shown that the dialysates of 10% homogenates of liver, heart, and kidney produced images and that their formation was also inhibited by iodoacetic acid. This indicated that the substance responsible for the image was of lower molecular weight than protein. A 10% homogenate of the liver in distilled water was

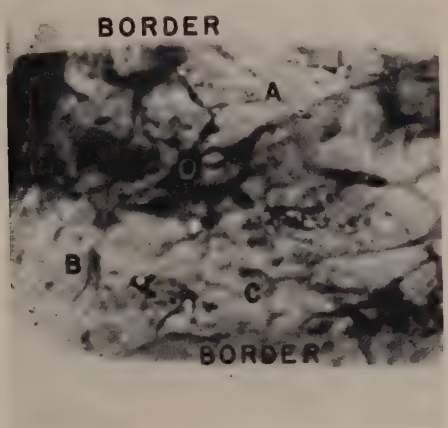

Fig. 2 An image of guinea pig liver after exposure of one hour, showing the progressively widening border and areas suggestive of liver lobules. (Area A: lobules are outlined and portal veins are seen. Areas B and C: poorly outlined but showing central veins.) × 7.

Images of longer exposure were so diffusely blackened that morphological details were less evident.

boiled for 10 minutes to prove thermo-stability of the substance. Again image formation remained.

The results of a paper chromatography experiment showed that a 76% alcoholic extract of guinea pig liver, which had the ability to strongly reduce the NTB plate, contained relatively large quantities of glutathione. This observation, together with the rather universal distribution of the image-producing substance throughout the tissues, and its dialyzability, thermostability, and inhibition by iodoacetic acid strongly suggested the presence of sulfhydryl compounds, such as cysteine and glutathione.

3. Chemical agents. Distinct images were produced by pure solutions of glutathione and cysteine (10^{-2} and 10^{-3} molar) but not by their oxidized forms. The density of the images produced by serial exposures of glutathione in 10^{-3} molar solution increased up to 6 hours and then lessened (fig. 3), as did the densities of the liver images (fig. 1).

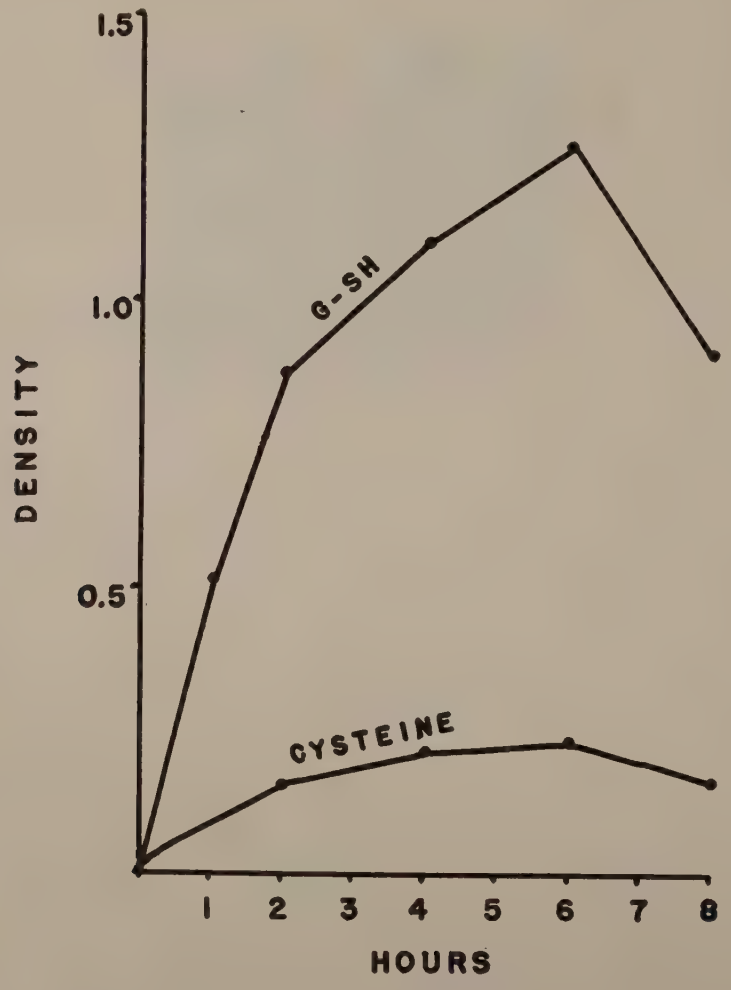

Fig. 3 Image densities of 10^{-3} molar solutions of glutathione and cysteine plotted against time.

It might be expected that all sulfhydryl compounds would produce images proportional in density to the concentration of — SH groups. This was not found to be the case. Although the solutions were calculated to produce the same concentration of — SH groups, it is seen (fig. 3) that the density of the cysteine image is approximately one-fifth that of glutathione. The gross and the microscopic views of the images produced by these substances also differed considerably as outlined in table 2. This perhaps is explained by the different solubilities of the substances in aqueous contact phase.

Compared with glutathione and cysteine, dimercaptopropylurea, a substance containing two — SH radicles in the molecule, did not give a black image, but one which was grossly yellow with a faint black border. Microscopically the image was composed of many tiny yellow grains tightly packed with scattered black grains of larger size presenting a "salt and pepper" appearance (table 2). The yellow of the image may have been produced by either manner in which light is reflected through the silver grains or by oxidation products deposited in the gelatin (Mees).

Various other pure compounds were then tested in 10^{-2} and 10^{-3} molar solutions. No image was produced by any of the following substances: methionine, glutamic acid, glycine, glucose, glycogen, lactose, arabinose, urea, uric acid, acetone, formaldehyde, formic acid $(NH_4)_2HPO_4$, $(NH_4)_2SO_4$, NH_4Cl, potassium nitrite, sodium sulfite, sodium nitrite, cholesterol, ethyl alcohol, ether, xylene, water, $HgCl_2$, $CuSo_4$, iodoacetic acid, p-chloromercurobenzoic acid, NaF, malonic acid, KCN, or phosphate buffer components.

Because of its well-known ability to reduce silver (Barnett and Bourne), ascorbic acid was considered as one of the more likely biochemical substances to be found in the tissues mentioned which might explain part of the phenomenon. Ascorbic acid in 10^{-2} and 10^{-3} molar solutions was then tried directly on the plate using the method described above. The pH was varied from 3 to 7.6 without any positive reaction.

Glutathione was detectable with the NTB plate in solution ranging from 10^{-1} to 10^{-5} molar; however, its capacity to form dense images was greatest at 10^{-3} molar solutions. With 10^{-1} and 10^{-5} molar solutions, images were grossly observable but were weak. Correspondingly, it was observed that glutathione had the ability to densitize [2] least in a 10^{-4} molar solution and greatest in 10^{-1} molar and 10^{-6} molar solutions (table 3).

TABLE 2

Comparison of images formed by three sulfhydryl compounds

	GLUTATHIONE	CYSTEINE	DIMERCAPTO-PROPYLUREA
1.	Images appear grey.	Images appear black.	Images yellow with black border.
2.	Small grains which are black or translucent.	Grains tightly formed into spicules. Look like iron filings. None translucent.	Tiny grains, yellow and black, which give "salt and pepper" appearance.
3.	Grains found throughout emulsion.	Grains on one plane.	Grains on one plane.
4.	Forms silver mirror about periphery of image.	No silver mirror.	No silver mirror.
5.	Gross and microscopic dots which increase in diameter with exposure time.	No dots.	No dots.
6.	Oxidation border increasing in width with increased exposure	No border.	Faint black border.

The microscopic observations recorded under 2, 3 and 5 were made at 970 ×.

[2] Desensitization was tested for by exposure to the flash of an incandescent light after exposure to sponges containing the glutathione.

DISCUSSION

It is generally believed that glutathione has a wide distribution in cells of most tissues; in fact, such tissues as rat liver, kidney, heart, spleen, muscle and blood have been measured for reduced glutathione by different methods, and rat liver has been shown to contain more glutathione than the other tissues mentioned (Woodward). If we are to assume that, within limits, the densitometer readings presented in this report correspond to the concentration of glutathione in the organ slices, and that the concentration found in the rat is comparable to the concentration in the guinea pig, table 1 may be of interest. Images of liver are the most dense, those of heart and kidney less so, and those of muscle and spleen least

TABLE 3

Effect of varying concentration of glutathione solution on image density and desensitization

Molarity of solutions	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Positive image density	.19	.82	1.12	.53	.22	0
Desensitization: Background image-density Minus desensitized area	.78	.62	.59	.10	.20	.78

dense. The order of magnitude is roughly correct, except for the spleen which by both the manometric and the titrimetric methods of estimating reduced glutathione (Schroeder and Woodward) was found to be second only to the liver. This may be explained by the fact that the red blood cells of the spleen were measured by these methods; however, unless hemolyzed, the red cells produced only a faint image on the NTB plate and therefore may account for the increased density by the methods mentioned.

Since iodoacetic acid inhibited the image produced by tissue slices and homogenates, the sulfhydryl compounds were logically implicated in the phenomenon. The physical properties of the substance in liver homogenate were similar to

those of glutathione; i.e., it was dialyzable, thermostable and able to combine with iodoacetic acid. Results of the paper chromatogram proved glutathione to be present in relatively large quantities. The image produced by glutathione had a distinct border, increasing in width with increased exposure time, which did not occur in the case of cysteine. This "border" phenomenon was also observed in the case of the liver images. Grossly observable black dots which increased in diameter with increasing time of exposure (table 2) were also found in images of glutathione and liver. Glutathione images likewise produced a silver mirror about their periphery, whereas those of cysteine did not. It appears reasonably probable that the reaction of the guinea pig liver slices was produced mainly by glutathione; however, it may be inaccurate to regard the entire picture as being caused by glutathione, since no understanding of the effect of other sulfhydryl-containing compounds (glutamylcystein, cysteinylglycine, and protein-bound — SH) is at hand.

SUMMARY

A variety of guinea pig tissue slices were shown to produce images upon the NTB emulsion. It was found that the hypothetical image-producing substance of the liver was dialyzable, thermostable, and could be inhibited by iodoacetic acid. Solutions of ascorbic acid, glucose, cystine, methionine and many other compounds failed to produce images; but solutions of pure glutathione, cysteine, and dimercaptopropylurea were effective. A resemblance between the images of glutathione and liver is noted.

ACKNOWLEDGMENT

The original observation, that bone marrow of a "control rat from a C^{14} autoradiographic experiment fogged the NTB emulsion was made by G. A. Boyd. It was through his encouragement that the studies reported here were pursued.

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NUTRITION OF ANIMAL CELLS IN TISSUE CULTURE

V. EFFECT OF INITIAL TREATMENT OF CULTURES ON THEIR SURVIVAL IN A SYNTHETIC MEDIUM [1]

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Previous studies from this laboratory have reported the development of a synthetic feeding mixture (no. 199) that supports cell life for an average period of 4 to 5 weeks (Morgan, Morton and Parker, '50; Morton, Morgan and Parker, '50; Parker, Morgan and Morton, '50). In the experiments described, chick embryo mesenchyme tissues were cultivated directly on the inner surface of pyrex test tubes, without the use of fibrin clots, and the feeding mixtures were renewed at frequent intervals. The transfer of nutrient substances from the embryos was reduced to a minimum by the use of the smallest amount of tissue that would provide adequate growth areas. In the early experiments, the cultures were treated with a preliminary feeding solution containing horse serum and chick embryo extract, which was left on the cultures for three days while the cells were becoming established. On the 4th day, the preliminary feeding mixture was removed and replaced by a synthetic medium. All cultures were then maintained in synthetic media until living cells were no longer discernible.

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[2] With the technical assistance of Miss B. J. Barrett, Miss E. A. Burr and Miss O. E. Rockett.

Although these procedures gave consistent results, it was felt that the use of a preliminary feeding solution containing naturally-occurring ingredients was open to serious theoretical objections. Consequently, an extensive series of experiments was made in an effort to eliminate this preliminary period of cultivation in non-synthetic media. The results of these experiments are described in the present report.

MATERIALS AND METHODS

The tissues, obtained from the leg muscle of 11-day-old chick embryos, were chopped with cataract knives into extremely small fragments that were then washed in a balanced salt solution (Earle, '43). Small amounts of the resulting suspension were transferred with a pipette to the inner surface of standard pyrex test tubes (18×150 mm) and the medium was placed in the bottom of the tubes out of contact with the tissue fragments. After a suitable drying period, usually one hour, the tissue fragments were flooded with medium and the tubes were incubated at 38°C. in an almost horizontal position in a slowly-rotating rack.

Synthetic Mixture 199 consists of amino acids, vitamins, purines, pyrimidines, a source of fatty acid (Tween 80), certain intermediary metabolites and accessory growth factors, and a modified Tyrode's solution. Details of the synthetic mixture and the assay procedures have already been reported (Morgan, Morton and Parker, '50).

The non-synthetic preliminary feeding mixture, used in certain experiments, consisted of 4 parts horse serum, 6 parts balanced salt solution (Earle, '43) and contained final concentrations of 1% chick embryo extract (Morgan and Parker, '49) and 0.002% phenol red.

EXPERIMENTS AND RESULTS

A. Effect of frequency of renewal of synthetic Mixture 199 on the length of survival of cultures that had received a preliminary mixture containing serum and embryo extract. Tis-

sues maintained in the preliminary feeding mixture might conceivably store nutrients derived from the serum or embryo extract. These reserve nutrients would then influence the length of survival of the cells during their subsequent cultivation in synthetic media. To test the magnitude of this effect, if it existed, a large group of cultures was prepared and cultivated in serum and embryo extract for three days. At the end of this time, the cultures were divided into groups and all were supplied with synthetic Mixture 199, but the

TABLE 1

Effect of frequency of renewal of synthetic Mixture 199 on the length of survival of chick embryo mesenchyme cells in roller tubes after an initial starting period of 3 days in a medium containing serum and embryo extract

FREQUENCY OF RENEWAL (MIXTURE 199)	NO. OF CULTURES [1]	AVERAGE SURVIVAL AFTER INITIAL STARTING PERIOD
		days
3 times a week	30	33
Twice a week	21	37
Once a week	27	50
Once every 2 weeks	29	48
Once every 3 weeks	20	47
After 4 weeks [2]	37	32

[1] Combined data from 5 separate experiments, each of which included cultures in three or more of the groups listed.

[2] Few cultures survived beyond this period.

frequency with which the culture fluids were renewed varied from three times a week to once a month. The results are shown in table 1. No significant difference in the average period of survival was observed between groups of cultures whose fluids were renewed three times and twice a week, respectively. But cultures whose fluids were renewed once a week, once every two weeks or once every three weeks lived almost half as long again as did cultures whose fluids were renewed three times a week. It was also observed that cultures whose fluids were renewed less frequently than once every three weeks rarely survived longer than one month.

B. Effect of length of initial starting period in synthetic Mixture 199 on the survival time of the cultures. The results of the previous experiments suggested that the presence of tissue break-down products in the medium *and/or* the cultivation of the cells in a preliminary feeding mixture containing naturally-occurring ingredients very probably contributed to their longer survival in synthetic medium. Accordingly, attempts were made to eliminate entirely the preliminary treatment with serum and embryo extract. Cultures were prepared as before and were supplied immediately with synthetic Mixture 199. After three days, this medium was removed and replaced three times a week. But cultures treated in this manner did very poorly and could seldom be used as test material. It was found, however, that when the initial period in synthetic medium was lengthened the cultures became better established and survived for much longer periods (table 2). When the initial period in Mixture 199 was extended to

TABLE 2

Effect of initial treatment on the length of survival of chick mesenchyme roller-tube cultures subsequently maintained in synthetic Mixture 199

INITIAL TREATMENT [1]	NO. OF CULTURES [2]	AVERAGE SURVIVAL AFTER INITIAL STARTING PERIODS
		days
Cultures held 3 days in medium containing serum and embryo extract	30	33
Cultures held 3 days in Mixture 199	59	10
Cultures held 7 days in Mixture 199	21	19
Cultures held 14 days in Mixture 199	22	28

[1] After these initial periods, all cultures were maintained in Mixture 199, which was removed and replaced three times a week. Volume of medium was 1.5 ml per culture.

[2] The various culture series comprising these experiments were not carried simultaneously.

two weeks, the cultures survived almost as long as when they were treated for a three-day initial period in a medium containing serum and embryo extract.

C. Effect of volume of medium on the usefulness of cultures after an initial starting period of 5–7 days in synthetic Mixture 199. The results of the experiments just described indicated that cultures held for an initial period of two weeks in synthetic medium before regular fluid changes were begun survived for a longer time than did cultures held for only one week under similar conditions. It was of interest, therefore, to determine whether any relationship exists between

TABLE 3

Effect of volume of roller-tube cultures after an initial starting period of 5 to 7 days in synthetic Mixture 199

VOLUME OF MEDIUM	NO. OF CULTURES [1]	USABLE CULTURES	GOOD CULTURES
ml		%	%
0.5	31	45	15
0.75	79	60	27
1.0	28	61	6
1.5	79	47	12

[1] Combined data from 4 separate experiments.

the volume of medium placed on the tissues and the length of this initial period. In all previous experiments, 1.5 ml of medium was added to each culture and the same volume was employed during the subsequent renewals of fluid. Experiments were now carried out in which cultures containing approximately the same amount of tissue were divided into 4 groups that received 0.5 ml, 0.75 ml, 1.0 ml and 1.5 ml of Mixture 199, respectively. At the end of 5–7 days, all cultures were examined under the microscope and were graded according to the amount and appearance of the tissue (table 3). It was found that by using 0.75 ml of medium it was possible to achieve a higher percentage of good cultures than was obtained with either larger or smaller volumes.

D. Effect of volume of medium and length of starting period on the survival time of cultures in Mixture 199. Since better cultures were obtained by decreasing the volume of synthetic medium used during the starting period, further experiments were carried out in which variations were made both in the volume of medium and in the length of the preliminary period. Comparable groups of cultures, all containing approximately the same amount of tissue, received either 0.75 or 1.5 ml of Mixture 199 and the medium was left without renewal for either one or two weeks. At the end of this time

TABLE 4

Effect of volume of initial medium and length of starting period on the length of survival of chick mesenchyme roller-tube cultures in synthetic Mixture 199

STARTING PERIOD IN MIXTURE 199 [1]	VOLUME OF MEDIUM	NO. OF CULTURES [2]	AVERAGE SURVIVAL AFTER INITIAL STARTING PERIODS
	ml		*days*
1 week	0.75	9	37
	1.5	21	19
2 weeks	0.75	5	34
	1.5	22	28

[1] After the initial starting period, all cultures received 1.5 ml of Mixture 199, which was subsequently removed and replaced three times a week.

[2] Only two experiments included cultures in two or more of the 4 groups.

all cultures received 1.5 ml of Mixture 199, which was subsequently removed and replaced three times a week. The results showed that by decreasing the volume of the initial medium to 0.75 ml the cultures survived for longer periods (table 4). This effect was obtained with cultures held for both one and two weeks, although the difference was greater when the cultures were held for one week only. It should be pointed out that cultures maintained in 0.75 ml of Mixture 199 for one week with subsequent renewal of the medium (1.5 ml) three times a week survived somewhat longer than cultures started with serum and chick embryo extract and then transferred to Mixture 199 (see table 2).

E. Procedures finally adopted for use with Mixture 199. The results of these various experiments, which involved more than 1200 cultures, have shown that preliminary treatment of the tissues with a medium containing naturally-occurring ingredients is no longer necessary. Consequently, all roller-tube cultures in this laboratory that are supplied with synthetic mixtures are now started directly in 0.75 ml of the mixture, which is left on the cultures, without renewal, for 5 to 7 days. At the end of this time, the cultures are examined microscopically and unsuitable cultures are discarded. The remaining cultures are then divided into the number of groups required in the proposed experiment and the 0.75 ml amounts of synthetic medium used as the starting mixture are removed and replaced by 1.5 ml of the various test media, which are then renewed twice a week for the duration of the experiment. The several hundred cultures that have been treated with Mixture 199 according to this modified procedure have survived for periods that are almost identical with those of cultures treated for a three-day initial period with a medium containing serum and embryo extract.

DISCUSSION

In all tissue culture experiments in which synthetic media are employed, the transfer of nutrient substances along with tissue explants cannot entirely be controlled. This transfer is particularly serious when embryo tissues are used, since embryo extract is a potent source of growth-promoting substances (Carrel, '13). Thus, White ('46) reported the cultivation of embryo tissues for 56 days in a synthetic medium, although his results were criticized by Fischer and his colleagues ('48) who suggested that sufficient quantities of embryo juice may have been introduced along with the explants to account for the results obtained. Also, Layton and Tutelman ('49) reported that the medium developed by White was inadequate for the growth and survival of tissue explants that had previously been washed free of adhering tissue juice.

In later experiments, White ('49) used smaller amounts of tissue and reported the survival of certain cultures for several weeks, but the culture that lived for the longest period (over 80 days) received embryo extract at about the 8th week.

Once the carry-over of embryo tissue juice has been reduced to a minimum, it becomes extremely difficult to obtain suitable cultures when they are started directly in the present synthetic mixtures. The results of extensive experiments, summarized in table 2, showed that cultures could be started directly in synthetic media provided the initial medium was left on the cultures, without renewal, for a period of 5 to 7 days. It was also found that better cultures were obtained when the volume of medium was decreased from 1.5 to 0.75 ml during this initial period (table 3). At the end of the 5- to 7-day starting period, adequate growth areas were established and the normal volume of medium (1.5 ml) could be added and subsequently renewed at frequent intervals. Actually, this procedure is probably effective for the same reasons that the three-day treatment with serum and embryo extract is effective. During the initial 5- to 7-day starting period, the small quantity of synthetic medium now used to bathe the tissue fragments undoubtedly accumulates a relatively high concentration of cell break-down products, which, in turn, are utilized by the cells while they are becoming established in their new environment. Eventually, as the synthetic media are improved, either chemically or physically, or both, it may be possible to establish cultures in them quite as easily as in natural media.

During long-term cultivation of tissues in synthetic media, certain factors other than the composition of the medium become important. The initial tissue fragments gradually break down and are replaced by thin sheets of cells which, in turn, eventually degenerate and die. Thus, during the period of cultivation there is a continuous process of cell degeneration and cell death accompanied by autolysis of the dead cells and the release of intracellular material, presumably both protein and non-protein in nature. If the mass of original tissue is

large enough, this release of intracellular material could conceivably supply sufficient nutritive substances to support cell life for a considerable period, even though the medium itself might be completely inadequate. From these considerations, it would be expected that the less often the synthetic mixtures bathing the cultures are changed the longer the cultures would survive. This is borne out by the results shown in table 1. When the synthetic mixtures were changed once a week, the cultures survived about half as long again as when the mixtures were changed two or three times a week.

It does not follow that all changes produced in the medium by the tissues are undesirable, since metabolic products from living cells and autolytic products from dead cells may contribute significantly to survival of the cultures. An illustration of this effect is the use of a "conditioned" medium by Sanford, Earle and Likely ('48) in the derivation of pure strains from single, isolated cells. But in attempts to develop an adequate synthetic medium, it seems advisable to reduce the conditioning effect to a minimum and this can be done most effectively by frequent replacement of the feeding mixture. In this way, the survival of the cells can be related directly to the composition of the medium and not to the ability of the cells to modify and improve that medium. It should be pointed out, however, that a synthetic mixture devised by a technique involving frequent renewals of medium will not necessarily be adequate if the culture fluids are renewed less often. Under conditions of infrequent renewal certain ingredients of the mixture may become depleted.

The more often culture tubes are opened for renewal of the medium the greater is the danger of bacterial or mold contamination. Blood plasma and serum, used in the traditional tissue culture media, have weak bactericidal properties that help suppress contaminations. Synthetic mixtures, on the other hand, are devoid of these properties and are rich in substances that promote microbial growth. In fact, the danger of contamination has become much more serious as the me-

dium has been enriched by basic nutrients and growth factors and as the assay period has lengthened. It is possible that contaminations might be suppressed by the addition of antibiotics, but until more precise knowledge of the effect of antibiotics on cell metabolism is available, the use of these agents would seem to be open to serious objections. The solution, at present, would appear to lie in the use of careful techniques and large numbers of cultures, so that a few may be lost without affecting, too greatly, the significance of the results. In this laboratory, it has been found helpful to renew the media twice a week instead of three times, as was done in the early phases of the work. This reduction in the amount of handling does not affect, appreciably, the length of survival of the cultures, but it does reduce by one-third the chances of contaminations and lessens considerably the maintenance work.

The results reported in the present communication suggest several technical procedures that would seem to be desirable in the development of synthetic media: (1) the carry-over of nutrient substances from the organism should be reduced to the lowest possible level by washing the tissue fragments to remove adherent tissue juices and by using the smallest amount of tissue that will provide suitable cultures; (2) the tissues should be cultivated directly in the synthetic medium without preliminary treatment with serum, plasma, embryo extract or other naturally-occurring ingredients; (3) the synthetic medium should be renewed at frequent intervals to eliminate the break-down products of dead cells and the metabolic products of living cells. If these conditions are fulfilled, an adequate synthetic medium for animal cells in tissue culture might be defined as one that will support unlimited cell growth and multiplication without the addition of even small amounts of naturally-occurring ingredients. Mixture 199 is not, in this sense, a completely adequate medium but it is useful as a maintenance medium for short-term experiments in cell

metabolism and as a basal solution for the study of additional substances of nutritional interest.

SUMMARY

1. A study has been made of the effect of variations in initial treatment upon the survival of chick embryo mesenchyme tissues cultivated in roller tubes in a completely synthetic medium (Mixture 199).

2. Tissues can be started directly in the synthetic medium if it is left on the cultures, without renewal, for a period of 7 to 14 days. It is possible to shorten this starting period to 5 to 7 days by reducing the volume of initial medium.

3. Cultures started directly in the synthetic medium survive for approximately the same length of time as cultures started in media containing serum and chick embryo extract.

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PURIFICATION OF CYPRIDINA LUCIFERASE

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ONE FIGURE

The luminescence of the marine crustacean, *Cypridina hilgendorfii*, results from the oxidation by molecular oxygen of a compound, luciferin, catalyzed by a specific enzyme, luciferase (Harvey, '40). To date, the participation of other cellular components in this reaction has not been demonstrated. Since it has been shown in the firefly that factors other than luciferin and luciferase are essential for light production (McElroy, '47; McElroy and Strehler, '49), the question arises as to homogeneity of the luciferase preparations obtained from *Cypridina*.

The luciferin of *Cypridina* has been partially purified (Anderson, '35), and its spectral characteristics before, during, and after light emission have been described (Chase, '43; Chase and Brigham, '51). Kinetic and other studies indicate that only one component in the luciferin preparations is essential for light emission. It has not been demonstrated, however, that there is only one component essential for light production in the luciferase preparation since all previous work has been done with crude aqueous extracts of the organism, with or without dialysis. The present report deals with the purification of luciferase from *Cypridina* and a study of the luminescent characteristics of the reaction carried out with the two purified components, luciferin and luciferase. The results indicate that only two components, as extracted from dried *Cypridina*, luciferase and luciferin, are necessary for light emission.

[1] This research was supported in part by funds of the Eugene Higgins Trust allocated to Princeton University.

EXPERIMENTAL PROCEDURES

The luciferase activities of the various fractions obtained in the purification procedure were determined by measuring the relative first order velocity constant of the luminescent reaction, using the photoelectric method of Anderson ('33). The velocity constant has been demonstrated to be proportional to luciferase activity (Chase, '50).

The luminescent reactions were carried out at room temperature in 0.067 *M* phosphate buffer, pH 6.8, containing 0.01 *M* NaCl. The following procedure was adhered to in all of the experimental runs: Immediately prior to testing a particular luciferase preparation, 0.10 ml of luciferin stock solution [2] was placed in the reaction vessel of the light measuring apparatus, and 10 ml of phosphate buffer were immediately added. The enzyme preparations were appropriately diluted (as indicated from approximate visual estimates of their activities) and 0.2 to 2.0 ml were added, in sufficient buffer so that the final volume of the luciferin-luciferase mixture would be 20 ml. Light emission was recorded until the reaction was completed, and the relative first order velocity constant was calculated for each luciferase fraction.

Protein determinations on the various luciferase fractions were made by the method of Herriott ('41), as modified by Lowry (unpublished).

RESULTS

Purification of luciferase. A crude aqueous extract of *Cypridina*, containing an active luciferase, can be prepared by grinding the dry organisms in a mortar and then extracting with water. In the following purification procedure, 20 gm of the organisms were first ground in a mortar, and then with

[2] The final product from Anderson's ('35) purification procedure for luciferin is in n-butyl alcohol, saturated with hydrogen. To prepare the luciferin stock solution used in the present experiments, the butanol was removed, *in vacuo*, from 0.75 ml of this luciferin solution. The residue, containing the luciferin, was then taken up in 1.5 ml of 0.1 *N* hydrochloric acid and this stock solution was kept in an ice water bath in order to retard loss of the luciferin by spontaneous oxidation.

water in a Ten-Broeck tissue grinder. The first extraction was made with 100 ml of H_2O. After centrifuging in the cold at 3000 RPM, the residue was re-extracted with an additional 50 ml of H_2O. The combined supernatants were made up to a volume of 150 ml; then cooled to 10°C. and the pH rapidly adjusted to 4.5 with I N HCl. The resulting precipitate was immediately removed by centrifugation in the cold, and the pH of the supernatant adjusted to 7.5 with I N NaOH. The clear amber supernatant was cooled to zero degrees and cold acetone was slowly added to 35% saturation. The mixture was then kept at approximately —5°C. for 15′, and the resulting precipitate discarded after centrifugation in the cold. Additional cold acetone was added to the supernatant to raise the saturation to 55%. The temperature was kept near zero at all times. The mixture was kept at 0°C. for 30 minutes after the final addition of the acetone, and the resulting precipitate was removed by centrifugation, and dissolved in 60 ml of H_2O (fraction I). Further fractionation was carried out with $(NH_4)_2SO_4$. The pH of 50 ml of fraction I was adjusted to 7.5, and solid $(NH_4)_2SO_4$ was added to 40% saturation. The pH was maintained at 7.5 with NaOH. After cooling at 0°C. for 15 minutes, the precipitate was removed by centrifugation and discarded. Solid $(NH_4)_2SO_4$ was added to raise the per cent saturation to 65, while the pH was maintained at 7.5. The mixture was cooled for 20 minutes at 0°C. and the precipitate was removed and dissolved in 15 ml of water (fraction II). Further purification was achieved by adsorbing onto and eluting from calcium phosphate gel. Five milliliters of calcium phosphate gel (1.6% by weight) were added to 15 ml of fraction II, and the pH was adjusted to 7.0. The mixture was gently stirred for 10 minutes at approximately 5°C. after which the gel was removed by centrifuging in the cold. The pH of the supernatant was maintained at 7.0 and 60 ml of calcium phosphate gel were added. After standing for 15 minutes, the gel was removed by centrifugation. All of the luciferase was removed from the supernatant by the latter procedure, but could be partially

recovered from the gel by eluting with alkaline $(NH_4)_2SO_4$. The gel was vigorously stirred for 5 minutes with 25 ml of 10% $(NH_4)_2SO_4$ (pH 7.3) and the mixture allowed to stand for 30 minutes at 5°C. The gel was removed by centrifugation, and once again treated with 25 ml of alkaline $(NH_4)_2SO_4$. The first eluate was fractionated with $(NH_4)_2SO_4$ in the same manner as described for fraction II. The resulting precipitate (40–65% $(NH_4)_2SO_4$ fraction) was dissolved in 5 ml of

TABLE 1

Purification of Cypridina luciferase

FRACTION	MG PROTEIN	RELATIVE RATE CONSTANT		SPECIFIC ACTIVITY RATE/MG PROTEINS	RECOVERY PER CENT
		k/ml	k × Dilution		
Crude Extract	14.0	1.56	156	11	100
Fraction I (0.35–0.65 sat. Acetone)	2.4	0.81	162	68	46
Fraction II (0.40–0.65 sat. $(NH_4)_2SO_4$)	1.1	4.46	446	405	32
Fraction III (Calcium Phosp.– elution)	0.08	1.00	100	1250	2.5
Fraction IV (Calcium Phosp.– elution)	0.03	0.46	46	1533	1.5

The preparation of the various fractions and method of enzyme assay are described in the text. The k values were obtained from a plot of log (a-x) vs. time. In all cases, except for fraction I, the enzyme preparations were diluted 100 times for assay. Fraction I was diluted 200 times.

H_2O (fraction III). The $(NH_4)_2SO_4$ saturation of the second eluate was immediately raised to 65%, and the resulting precipitate was dissolved in 5 ml of water (fraction IV).

The activities of the various fractions are presented in table 1. The results indicate that several different procedures may be used in the purification of the enzyme. Repeated $(NH_4)_2SO_4$ precipitation of fraction II has given preparations with a specific activity 100 times over the crude and with much higher recoveries than that obtained with the calcium phosphate treatment.

Stability of enzyme. An analysis of the temperature inactivation of dialyzed, crude luciferase solutions has been presented by Chase ('50). Preliminary experiments performed on fraction IV from the present purification procedure for luciferase (see fig. 1) indicate an increased stability at lower temperatures, although the same mechanism of heat inactivation seems to exist as reported previously for the relatively crude enzyme. The latter is inactivated at 40°C. to about the same extent as is fraction IV at 47°C. At 50°C., however,

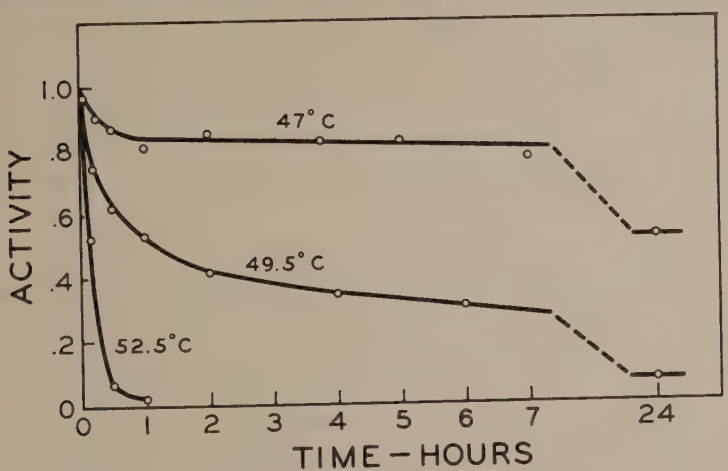

Fig. 1 Course of inactivation of purified luciferase (fraction IV) at different temperatures. The method for determination of relative enzyme activity after heat treatment is the same as that described in the text (Experimental Procedure) for the assay of the various fractions from the purification procedure.

fraction IV and the crude enzyme solution show similar characteristics. It is probable that impurities in the relatively crude preparations used in the earlier work affected the temperature stability of the enzyme, and a repetition of some of the experiments with more highly purified luciferase is certainly desirable.

Fraction IV is quite stable in the frozen state, and has been kept in this condition for over three weeks, with little or no loss of activity. It is also quite stable for at least two weeks at 4°C.

Requirements for metal ions. No requirements for the usual inorganic ions, such as Mg^{++} or Mn^{++} have been observed for the purified enzyme preparations. In addition, crude luciferin preparations appear to be no better than the purified luciferin, insofar as relative light intensity is concerned.

Specificity of luciferases. When purified *Cypridina* luciferase is mixed with purified firefly luciferin, no light is emitted, even in the presence of adenosine triphosphate. Likewise, firefly luciferase fails to stimulate the luminescent oxidation of *Cypridina* luciferin. The results indicate a high degree of specificity of the luciferins and luciferases from luminous forms. Among the fireflies, however, it has been clearly demonstrated that luciferin from the beetles of one genus gives light with the luciferase from another genus. Presumably the same mechanism of light production exists in these forms (McElroy and Harvey, '51).

Absorption spectrum. The ultraviolet absorption spectra of the crude extract and of fractions I, II, III and IV were measured with the Beckman spectrophotometer. A consistent difference was found between the spectra of the fractions possessing a high and those possessing a low specific activity. The former tended to have an absorption maximum at about 275–280 mμ while the latter (low specific activity) had a band centering more nearly at 265 mμ. It would appear that luciferase activity is not related to purine or nucleic acid components.

DISCUSSION

The present results indicate that in extracts from *Cypridina*, only two components are essential for light production, luciferin and luciferase. The enzyme which has been purified over 150 times is just as effective as, and shows characteristics similar to, the crude cold water extract. The results which have been obtained with firefly extracts indicate that a fundamental difference exists between the light emitting process of these two forms. A closer analysis, however, of the conditions of the experiments in the two cases does not exclude the

possibility that a similar process is operative. In *Cypridina*, the luciferin is extracted from the *dried* organisms under conditions where no light emission is observed. From this extract a labile substance (luciferin) is partially purified and causes light emission when mixed with purified luciferase. In the firefly, however, extracts are made from *fresh* tissue under conditions where luminescence is complete, and presumably all of the active luciferin has been destroyed. This dark preparation will emit additional light, however, if adenosine triphosphate is added. It has since been shown that reduced luciferin is still present in these preparations (McElroy, '51). This reduced luciferin can be oxidized, but light is emitted only in the presence of ATP. In addition, with ATP in excess, the total light obtained depends upon the amount of reduced luciferin present, making it comparable to the *Cypridina* system. The possibility exists, therefore, that in the dried *Cypridina* a highly labile energy-rich luciferin is preserved, which will subsequently emit light in the presence of luciferase. Apparently, the system concerned with the resynthesis of *Cypridina* luciferin has been inactivated during the drying and storage of these organisms. Preparations from fireflies which have been dried and kept at room temperature gradually lose their ability to respond to ATP, although a very bright light is obtained during the extraction with water. The results appear to be comparable to the *Cypridina* system, suggesting that in the fireflies a labile component is still present in these dried preparations, which will luminesce.

ACKNOWLEDGMENTS

It is a pleasure to acknowledge the capable assistance of Miss Elizabeth H. Brigham who assayed the enzyme preparations and Mr. Howard Lenhoff who assisted in the preliminary experiments on purification. We would like to thank Prof. E. N. Harvey for his continued interest and suggestions during the course of these experiments.

SUMMARY

1. The luciferase from *Cypridina hilgendorfii* has been purified over 150 times by means of $(NH_4)_2SO_4$ and acetone fractional precipitation and adsorption onto and elution from calcium phosphate gel.

2. Preliminary experiments indicate that the purified luciferase is more resistant to heat inactivation, at least at the lower temperatures, than is the crude enzyme. The kinetics of the inactivation process, however, appear to be the same for both preparations.

3. Mixtures of both purified luciferin and luciferase emit a very bright light. Requirements for other components, such as Mg^{++} and Mn^{++} ions, could not be demonstrated.

4. The ultraviolet absorption spectrum of the purified enzyme showed a maximum at approximately 280 mμ.

5. The results are briefly discussed in relation to the general problem of light production in other forms.

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THE RIGIDITY OF THE CELL CORTEX DURING CELL DIVISION [1]

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ONE FIGURE

Presumably all living cells have a cortical region which is physically and chemically different from the cell interior. This differentiated cortical region has been reported for various types of cells. Recently also the skeletal muscle cell has been shown to have a cortex which is more rigid than the interior protoplasm (Rieser, '49). The cell surface and cell cortex have played a significant role in the development of biological thought and theory, and a number of prominent biologists have emphasized the importance of the cortex in cell processes (see, for example, Just, '39). Certainly there can be no doubt that the cortex does play a significant role in the life of a living cell.

During the course of cell division in the egg of the annelid worm, Chaetopterus pergamentaceus, it is possible to make a physical study of the protoplasmic cortex. For in this egg, granules can be moved from the cortex as a result of moderate centrifugal force. The force necessary to dislodge the granules varies during the mitotic cycle. Thus the relative changes in rigidity that occur in the cortex during the course of cell division can be determined. These changes are reported in this paper.

[1] This investigation was supported in part by a research grant from the National Cancer Institute, of the National Institutes of Health, Public Health Service.

METHODS

Eggs and sperm were obtained from the annelid worm, Chaetopterus pergamentaceus, by a method reported in an earlier paper (Heilbrunn and Wilson, '48). Eggs from one female were then poured gently into a small finger bowl containing about 50 ml of sea water at a temperature of 21.0°C. The finger bowl containing the eggs was then placed in a constant temperature bath maintained at a temperature of 21.0°C. A standard sperm suspension was prepared by adding two drops of "dry sperm" to 10 ml of sea water. Five or 6 drops of the standard sperm suspension were used to fertilize the eggs of one female.

At intervals between fertilization and cleavage, eggs were removed from the finger bowl and centrifuged in a hand centrifuge. The effective radius of turn of the centrifuge head was 6.8 cm., and the head of the centrifuge rotated 168 times per turn of the centrifuge handle. The centrifuge handle was turned either 40 turns in 60 seconds, 50 turns in 60 seconds, or 60 turns in 60 seconds. These rates represent a centrifugal force of 4000, 6250, and 9300 times gravity, respectively.

After centrifugation the eggs were removed from the centrifuge tubes, placed on a glass slide, and covered gently with a cover slip. The eggs were then observed under high power magnification.

In a Chaetopterus egg in which the granules of the interior protoplasm have moved to one end of the cell as a result of moderate centrifugal force, one can observe in the clear zone of the cell a layer of granules embedded in the cortex of the cell. This was observed many years ago by F. R. Lillie ('06). At various times after fertilization it is possible to determine the centrifugal force which will move an appreciable number of granules from the cortex. The end point used in these experiments was the centrifugal force, applied for one minute, required to disrupt the continuous string of granules in the cortex in at least 16 out of 20 eggs

but in less than 20 out of 20 eggs. For example, at 10 minutes after fertilization eggs were subjected to forces of 4000, 6250, and 9300 g. A force of 4000 g. and a force of 6250 g. were not sufficient to move the cortical granules in at least 16 out of 20 eggs, and a force of 9300 g. was more than sufficient to move the granules. The end point was taken to be between 6250 and 9300 g. With the equipment available it was not practicable to obtain gradations of centrifugal force between 6250 g. and 9300 g.

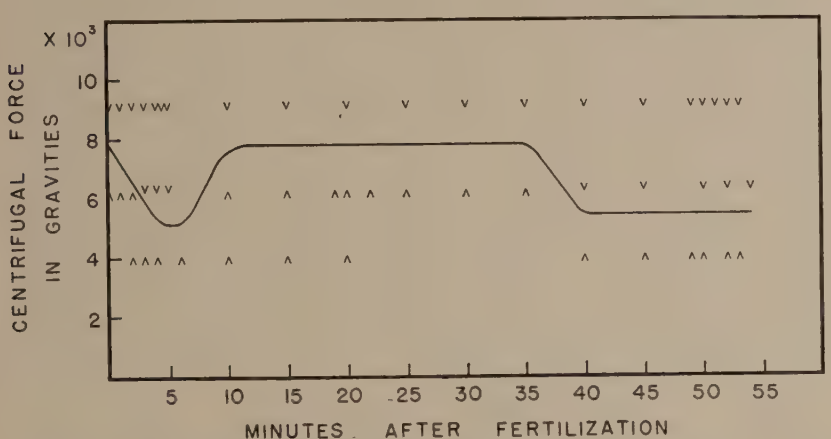

Fig. 1 The rigidity of the cortex of Chaetopterus during the time between fertilization and first cleavage. Cleavage time 54 minutes.

RESULTS

The results of the experiments are recorded in figure 1. In figure 1, a V represents a test in which the end point was exceeded, and an inverted V represents a test in which the end point was not reached. Immediately after fertilization the rigidity of the cortex begins to decrease. The decrease in rigidity stops at about 4 minutes after fertilization. At about 6 minutes after fertilization the rigidity begins to increase again. The rise is halted at about 10 or 11 minutes after fertilization. There is no further change in the rigidity until about 35 minutes after fertilization. At this time the cortical

rigidity begins to decrease again, and the fall is halted at about 40 minutes after fertilization. From this time until the cell cleaves there is no further change in the rigidity of the cortex.

DISCUSSION

An effort has been made to determine at various times during the mitotic cycle the relative changes in the state of gelation of the cortex during the mitotic cycle. Ordinarily one would express the degree of gelation of a substance in viscosity units. When one measures the speed of movement of a granule under the influence of a given centrifugal force, then one can apply Stokes' Law and get a value for the viscosity of a substance. In the present work due to the thinness of the cortical layer, it was not possible to determine the speed of the granules through a given distance of the cortex. As a matter of fact the movement of the cortical granules is largely through the interior protoplasm. Actually in the experiments reported in this paper what was determined was the shearing force necessary to dislodge the granules from the cortex, and I am not aware of the existence of a technical term which would apply to the results of these experiments. The terms rigidity, consistency, stiffness, and "viscosity" have been used by authors in the past to express the degree of gelation of the cortex when the cortex has been studied by dislodging the cortical granules by centrifugal force. In lieu of a better word I too have used the term rigidity.

Brown ('34) has reported that in the Arbacia egg the consistency of the cortex becomes much firmer as cleavage approaches. And this idea is shared by Marsland ('51). Brown and also Marsland ('39) determined cortical "viscosity" by the centrifuge method, and use as a method of "viscosity" change the variation in the width of the red pigment granule zone at the centrifugal end of the cell, assuming that the red pigment granules are contained in the cortex prior to centrifugation. However, the assumption that all of the red pigment granules are contained in the cortex is contrary to fact.

It can be shown very easily that many of the red pigment granules remain in the internal protoplasm throughout the time between fertilization and cleavage. If Arbacia eggs immersed in a drop of Ca-free sea water on a glass slide are covered with a cover glass, by applying pressure to the cover glass the eggs can be broken in such a manner that the internal protoplasm flows out through the ruptured cell surface into the external medium. If the eggs are observed with a microscope during the crushing process numerous red pigment granules can be seen in the internal protoplasm as it exudes into the external medium. Furthermore, Fischel ('06) has shown in the case of Arbacia pustulosa that in the time between fertilization and cleavage the pigment granules tend to migrate into the cortex. If this is true, then as time goes on fewer pigment granules can be moved through the egg by centrifugal force.

The results of the experiments reported in the present paper disprove the theory of Marsland, a modification of an earlier theory of Schechtman ('37), that a gelation of the cortical protoplasm plays a paramount role in the furrowing or cleavage process. Marsland has found that pressure applied to the fertilized Arbacia egg prevents cell division. According to Marsland the inhibition of cell division is due to the solation of the cortical protoplasm produced by the high pressure. Marsland has treated lightly the effect of pressure on the internal protoplasm of the Arbacia egg. He believes that "the pressure acts directly upon the cortical gel rather than upon concurrent chemical reactions in the cell" (Marsland, '38). However, the application of high pressure is not without effect on the internal protoplasm. In Echinarachnius high pressure causes the mitotic spindle to disappear (Seifriz, '24). Also in the egg of Urechis high pressure causes the dissolution of the spindle. It is possible that in Arbacia eggs high pressure has the same effect. This most certainly would prevent the egg cells from dividing. Furthermore, in the Chaetopterus egg there is no increase in the rigidity of the cortex prior to or during the cleavage process.

Comparing the relative rigidity curve of the cortex with the relative viscosity curve of the internal protoplasm of Chaetopterus (see Heilbrunn and Wilson, '48), it can be seen that the directional changes in both are similar except at the end of the cycle. In both the cortex and the interior there is a viscosity decrease following fertilization, and this decrease is followed by an increase and then another decrease. At the end of the cycle in the interior protoplasm there is an increase in viscosity as the cell undergoes cleavage; in the cortex there is no increase in rigidity during the cleavage process or prior to the cleavage process. Except at the end of the cycle it appears as if the cortex initiates the sol-gel changes in the cell.

SUMMARY

1. A method for determining the relative rigidity of the cell cortex is described.

2. Following fertilization there is a decrease in the rigidity of the cell cortex. At about 6 minutes (21.0°C.) after fertilization the rigidity of the cortex begins to increase, and the rise is halted at about 10 or 11 minutes after fertilization. The cortex remains in this condition until about 35 minutes after fertilization. At this time there is a decrease in cortical rigidity, and the fall is halted at about 40 minutes after fertilization. From this time until the cell cleaves there is no further change in the rigidity of the cell cortex.

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THE EFFECT OF X-RAY TREATED MEDIA ON HYDRA TENTACLES

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The irradiation of any metazoan cell in its natural environment necessarily subjects the protoplasm to substances produced by the irradiation of an aqueous medium in gaseous equilibrium with air. The effect of the irradiated medium may, therefore, be of considerable theoretical as well as practical importance. In most cases where the cell itself is irradiated it is difficult to distinguish between the effect of substances produced in the external environment of the cell; in the intracellular fluid; or an ionizing event occurring within a morphologically identifiable intracellular critical area.

An estimate of the effect of substances produced in the external environment may be made by subjecting the unirradiated cell or organism to the action of an irradiated medium. At present there is no way, known to us, to distinguish between the effects of substances produced intracellularly and those attributable to the activation of the "target theory" mechanisms.

In the present report we present data to show that x-ray irradiation produces in air saturated water or saline, substances which are deleterious to the morphological integrity and physiological function of tentacles of the common brown Hydra.

In an unfavorable situation, Hydra tentacles begin to show signs of degeneration by a clubbing at the distal ends as contrasted to the thin, pointed ends of the normal organs. As degeneration proceeds there is a progressive shortening and

thickening which continues until the attachment of the tentacle to the body is represented by nothing more than a slight bulge or thickening of the ectoderm. We have designated this stage as "stubs" and have taken it as the end of the reaction. The various degrees of shortening of the tentacles, the loss of contractility, and the sluggish response to physical stimulation are features which are readily observed yet are difficult or impossible to record quantitatively. The appearance of stubs, however, is unequivocal and for this reason we have chosen this morphological condition as indicating a reaction to the selected treatment.

Individual hydras, which had been grown as mass cultures in saline (NaCl, .05 gm; KCl, .002 gm; $CaCl_2$, .003 gm; H_2O, 1000 cm^3) and fed daphnia, were isolated in dishes containing 10 ml respectively of experimentally treated solution or control solution. At the time of isolation the number of tentacles on each hydra was recorded. Hydras having half or more of their tentacles reduced to stubs 24 hours after irradiation were recorded as a positive response.

All radiations were made with a beryllium window x-ray tube operated at 50 KV constant potential and 50 ma. with added filtration as described. Dose determinations were made with the chemical method of Andrews and Shore ('50) and are expressed in roentgens. The technique of this method of dosimetry gives, within the limits of its accuracy, the dose of x-ray absorbed by the fluids and not the air dose. All experiments were carried out in a room maintained at $23 \pm 2°C$. During irradiation the vessels containing solution were water cooled to about 15°C.

Two different types of vessel, held in a water-cooled support, were used to contain fluids to be irradiated. In one series of experiments the vessel was a Pyrex glass dish of approximately 100 ml capacity which contained 50 ml of fluid. This dish was loosely covered with an aluminum plate 0.022 inch thick which acted as a radiation filter. The distance from the exit port of the x-ray tube was constant for all experiments. In this type of vessel, hereafter referred to as an open dish,

there was an air volume of about 50 ml above the fluid. Our x-ray tube is mounted in a lead-lined box through which a rapid flow of room air is maintained. Although there is an air stream through the box, the air above the fluid in the open dish is essentially static. The second type of container was a Pyrex glass bottle of rectangular cross section and about 200 ml capacity which could be completely filled and closed with a ground glass stopper. There was no overlying air in the vessel. All of the solutions in this study were in equilibrium with room air. When using the closed flask the only filtration of the x-ray beam was that offered by the walls of the vessel.

Moderate doses of x-ray produce easily detectable quantities of hydrogen peroxide in water containing air. In this study hydrogen peroxide concentrations were determined by titration with potassium permanganate. We are indebted to Dr. W. R. Carroll of this Laboratory for making numerous titrations for us. Concentrations were determined shortly after irradiation and remained essentially unchanged during the experimental period. The hydrogen peroxide added to the experimental solutions was Merck's "Superoxol" and was used without further purification. All water in the experiments was double distilled, with second distillation being from Pyrex glass, and was used within 48 hours after the second distillation. No unusual precautions were taken to eliminate traces of dust contamination.

In the table the columns headed "Number of Hydra" are the accumulated totals of a number of experiments conducted over several days. For each experiment, irradiation of the required material was done just prior to use. In every instance both experimental and control systems were set up from the same culture of Hydra and from the same stock of fluid material.

In the experiments in which irradiated air was introduced into the saline, two procedures were followed. In the first a glass tube was fastened in the beam in such a manner that the open end was in the same position as that ordinarily occupied

by the vessel containing solution. The glass tube was connected by rubber tubing (shielded from the radiation) to a flask outside the x-ray box, containing 50 ml of saline through which the irradiated air in the box was drawn. The unfiltered output of our x-ray tube is 12,500 r per minute immediately adjacent to the window. In the first air experiment (no. 10, table 1) radiation was delivered to the static air of the lead-lined x-ray box at this rate for a total of 6 minutes. The dose given to air in the box was approximately 4,050,000 gram roentgens.

In the second method (exp. 11, table 1) an attempt was made to introduce into the saline a quantity of air comparable to that overlying the fluid in the open dishes. To this end an air-tight metal box fitted with suitable taps and with an aluminum cover 0.022 inch thick was made. This box and its contained room air, held in the position occupied by the vessels used to contain liquid, was irradiated for 12 minutes. After the irradiation the box was connected to a partially evacuated flask containing 50 ml of saline, and by slowly opening the outlet tap the irradiated air was allowed to bubble through the saline. Saline so treated was used as experimental fluid.

EXPERIMENTAL RESULTS AND DISCUSSION

Pertinent data from the series of experiments are presented in table 1. In the first experiment several hydras received 9,600 r (given in 6 minutes) in an open dish and each individual was then left for 24 hours in a separate portion of the saline in which it had previously been irradiated. Twenty-eight of the 30 animals treated showed stubs at the end of 24 hours.

In experiment 2 the hydras were irradiated as in the above test but immediately following the irradiation the animals were removed to individual portions of fresh unirradiated saline. Only three of the 30 animals so treated showed stubs at the end of 24 hours. These two experiments pointed quite

TABLE I

Effect of irradiated media on hydra tentacle degeneration after 24 hours' exposure to the media

EXPERIMENT NO.	TREATMENT	EXPERIMENTALS						CONTROLS	
		Irradiation time	Total dose	Molarity of H_2O_2	Air	No. of Hydra treated	No. of Hydra with stubs	No. of Hydra	No. of Hydra with stubs
		minutes	*roentgens*						
1	I[1] Hydra in I Saline	6.0	9,600	2.5×10^{-5}	Yes	30	28	15	2
2	I Hydra in U[2] Saline	6.0	9,600	2.5×10^{-5}	Yes	30	3		
3	U Hydra in I Saline	6.0	9,600	2.5×10^{-5}	Yes	30	24	18	0
4	U Hydra in I H_2O	6.0	9,600	2.5×10^{-5}	Yes	30	24	13	0
5	U Hydra in H_2O_2			2.5×10^{-5}		32	7	19	0
6	U Hydra in I Saline	6.0	7,500		No	28	3	35	2
7	U Hydra in I Saline	7.7	9,600	1.35×10^{-5}	No	30	0	30	1
8	U Hydra in I Saline	16.0	20,000	2.7×10^{-5}	No	30	3	30	5
9	U Hydra in I Saline	60.0	75,000	7.2×10^{-5}	No	20	2	16	0
10	U Hydra in U Saline and I Air	6.0			Yes[3]	20	5	20	0
11	U Hydra in U Saline and I Air	12.0			Yes[3]	24	5	26	0

[1] I = Irradiated; [2] U = Unirradiated; [3] See text for methods of irradiating air.

clearly to the influence of the medium on the observed results, and the other experiments in the series were designed to test the hypothesis that the medium was the more influential factor involved.

When the saline, as used for culture, was given a dose of 9,600 r in an open dish, 24 of 30 unirradiated hydras placed therein developed stubs in 24 hours. The same result was obtained when distilled water was irradiated with the same dosage and then salts added to make the usual culture saline.

Under our conditions of irradiation 9,600 r given to 50 ml of saline or water in an open dish produced a solution 2.5×10^{-5} molar in hydrogen peroxide. This concentration of peroxide added to unirradiated saline induced stubs in only 7 of 32 hydras treated.

In order to partially eliminate the effect of irradiated air, saline was irradiated in a closed flask and unirradiated hydras were placed therein. The results obtained following doses of 7,500, 9,600, 20,000 and 75,000 roentgens (1250 r per min.) are shown in the table as experiments 6, 7, 8 and 9. In the closed dish experiments, the number of individuals developing stubs was significantly less than that in the open dishes even when the concentration of peroxide in the former was greater than in the latter.

Two experiments were done to test the effect on Hydra of the products of x-ray treated air dissolved in saline. In one of the experiments (no. 10), 5 of the 20 hydras and in the second (no. 11) 5 of 24 developed stubs in 24 hours. Controls were observed in saline through which unirradiated air had been bubbled.

From the data presented it is obvious that the effect of saline given 9,600 r in an open dish is far greater than that to be expected from the concentration of hydrogen peroxide produced therein (2.5×10^{-5} molar). This is on the assumption that there is no difference in the effect of peroxide produced in the solution by radiation and the same molar concentration secured by adding "Superoxol." Furthermore, a

slightly higher concentration of peroxide, 2.65×10^{-5} molar, produced in saline by irradiation in a closed flask had little or no effect in the hydras. An explanation of the effect of 9,600 r in an open dish may be had if we assume that the result is due to a summation of the separate action of peroxide and products of the ionization of air dissolved in the solution. If this is the case one would expect that the sum of the action of the added peroxide and the irradiated air would not be far different from that found in the open dish. We find, however, that no matter how the results of the air and added peroxide experiments are summated there remains a large excess of action of the fluids irradiated in the open dish. It seems to us likely that an explanation is to be found by assuming that the continuing action of the radiation on the products of air ionization dissolved in saline in the presence of peroxide produces a group of highly toxic substances. There is no way to critically test this assumption since chemical methods for the quantitative determination of minute concentrations of suspected products of air ionization are not readily available.

The results of our experiments show conclusively that irradiation of either water or saline in a vessel of such construction that the solution is in contact with air produces in the solution a substance or substances which bring about the rapid degeneration of the tentacles of Hydra. It appears that the toxic action is not due entirely to hydrogen peroxide produced in the solution or to the ionization products of air dissolved in the solution or to a summation of these factors.

If one assumes that gram for gram the energy absorption of water and tentacle tissue is the same, it is clear that the dose of x-ray required to produce toxic media has little effect when applied directly to the tentacle. This would indicate that x-rays act on only certain cell constituents and that the dose used did not affect a sufficient number of such constituents to cause "stubbing" or that the concentration of products produced by irradiation of intracellular water, gas and salts

is not high enough to be effective or that such products are eliminated so rapidly that any change is transitory and, therefore, not readily observed. These possibilities, perhaps one should say probabilities, must be fully evaluated in proposing any mechanism for the action of radiation on cells or animals as a whole.

Many investigators have made an effort to distinguish between the effects of radiation which could be attributed to the indirect action through the medium and the effects readily explained by the target theory. In many of the experiments dealing with the effect of the irradiated medium it is impossible to evaluate the likelihood that substances other than hydrogen peroxide are produced during the irradiation of the medium.

Held and Bellamy ('50) show that irradiation of aquarium water with an increased oxygen content reduces the survival time and the productivity of female *Daphnia magna* over that found in normal water or water which had an increased nitrogen content. The authors do not give any figures for the concentration of hydrogen peroxide or other substances produced in their solution.

An increase in chromosome abnormalities produced by x-ray radiation when administered to the inflorescences of *Tradescantia* in an atmosphere containing an excess of oxygen has been shown by Giles and Riley ('50). Experiments such as these should generate speculation concerning the production of intracellular hydrogen peroxide and its effect on the cell.

Taylor, Thomas and Brown ('33) reported the production of media lethal to *Colpidium campylum* by x-ray treatment. They attribute their results to the presence of hydrogen peroxide, since the effect could be duplicated by adding this material to the medium. Protection was offered by 0.8% agar, two drops of 1.0% gelatin and two species of bacteria. The possible contribution of air to the results cannot be evaluated.

That non-specific proteins will protect against the effect of irradiated medium has been shown by a number of investigators: Evans, Slaughter, Little and Failla ('42) and Evans ('47). This would not necessarily mean that the active agent was hydrogen peroxide. Barron ('49) has suggested that the effective substances produced in sea water by irradiation may be organic peroxides. So far as we know no one has demonstrated the presence of such compounds in irradiated media.

SUMMARY

The data presented above show that:

1. When water or saline, in equilibrium with air, is irradiated with x-rays substances are produced in the fluids which bring about the degeneration of the tentacles of the common brown Hydra.

2. Saline irradiated with overlying air is more effective in producing tentacle degeneration than: (1) Irradiated air dissolved in unirradiated saline. (2) Saline irradiated without overlying air or (3) a summation of the effects of (1) and (2).

3. It is suggested that the increased activity of saline irradiated with an overlay of air is due to the continuing action of the radiation on the complex solution composed of irradiated water and irradiated air.

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CHANGE IN THE BIREFRINGENCE OF THE CORTICAL LAYER OF SEA-URCHIN EGGS INDUCED BY STRETCHING

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THREE FIGURES

A series of studies of cellular birefringence have been undertaken in an effort to correlate the changes in birefringence with the stresses and strains existing in dividing cells (Inoué and Dan, '51). The analysis is based upon the observation that the birefringence of ordinary gels is increased on extension and decreased or reversed in sign on compression.

Tests on several inanimate gels confirm this fact; the next step obviously requires the testing of structures in living cells whose gel nature has been satisfactorily proven. For this purpose the cortical layer of the eggs of the following echinoids was studied: the sea-urchins, *Pseudocentrotus depressus, Heliocidaris crassispina* and *Mespilia globulus*, and the sand dollar, *Astriclypeus manni*.

The polarization set up, as well as the method of causing doubly refractive objects to brighten and darken in alternate quadrants by means of a rotating mica plate, is the same as described by Inoué and Dan ('51). As is known from the work of Runnström, Monné and Broman ('44), the cortical layer of sea-urchin eggs has a birefringence which is positive in the direction of the radius, or, expressed differently, negative in the direction of the tangent. In the following paragraphs, observations are collected showing an increase in birefringence caused by stretching, and a decrease caused by slackening of the egg cortex.

(1) When unfertilized eggs are sucked into a fine capillary and stretched, the surface of the stretched part which is in contact with the inner surface of the capillary acquires a stronger negative birefringence (in the direction of the tangent), thus shining much more brightly than before (fig. 1). In contrast to this, the round ends either retain more or less the same brightness (in *Heliocidaris)* or become isotropic (in *Pseudocentrotus)*. However, if left stretched for some time within the capillary, the brightness of the linear part and the round ends is equalized.

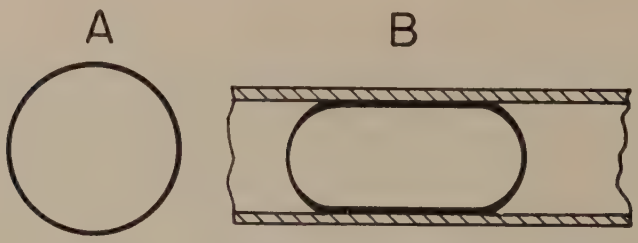

Fig. 1 Diagrammatic representation of the birefringence of the egg cortex. (A) Control egg. (B) Egg within a capillary. The thickness of the contour line is made proportional to the strength of the double refraction, showing the appearance of a stronger negative birefringence (in the tangential direction) at the stretched parts in contact with the capillary wall, and its weakening at the end parts.

The above situation remains the same even in the case of a capillary which has a relatively large bore, so that its diameter is only slightly smaller than that of the egg. Moreover, if the egg is blown out of the pipette, the inequality in birefringence is retained in the elongated cell lying free in the sea water *(Heliocidaris)*. If an egg is taken into a pipette of extremely small bore, the front end has a round contour, while the hind end takes a squarish shape in optical cross section (fig. 2). In such a case, no birefringence is apparent on the hind part. Now, if such an egg is gradually pushed out, the distribution of birefringence is reversed as the direction of its movement is reversed, again showing birefringence only at the new front end.

If an egg is pushed into a capillary which tapers rather abruptly, the cortical birefringence becomes continuously stronger toward the apex of the cone (fig. 3).

From the above facts it is obviously justifiable to consider that stretching increases and slackening decreases the cortical birefringence. It is of interest that this method can show local differences in the degree of stretching.

(2) If needles are stuck into an egg and the cytoplasm is pulled into two parts, the cortical birefringence becomes stronger on the stretched connecting stalk spanning the two

Fig. 2 An egg within a capillary of extremely small bore. At the hind end, the luminosity is practically non-existent.

Fig. 3 An egg pushed into a capillary which tapers steeply, showing the birefringence increasing toward the narrow, pointed end of the cell.

masses of cytoplasm. After the tearing of the stalk, as it shrinks back into the main mass of protoplasm, the birefringence becomes weaker and weaker and finally vanishes. Here again, a correlation between birefringence and tension is quite evident.

(3) Exovates are easily obtained by subjecting the eggs of *Pseudocentrotus* to a hypertonic medium (6 cm^3 of 2.5 M NaCl + 20 cm^3 of sea water) for some time and then returning them to normal sea water (see Just, '23). As the eggs swell to the original volume in the natural medium, they form exovates instead of swelling uniformly all around. If this process

is observed with the polarization microscope, it can be seen that the exovate acquires a stronger birefringence, while the luminosity of the remaining part disappears. In *Heliocidaris*, although the luminosity of the remaining part does not vanish completely, it certainly decreases in intensity. However, this difference in luminosity is eventually equalized if the exovate remains connected with the main body of the cell, as in the case of the capillary experiments. If an exovate is separated from the main cell body, the initial difference in birefringence is retained.

The result of fertilization of *Pseudocentrotus* eggs having exovates connected to them is rather interesting. Mr. T. Kajishima of the Laboratory kindly allowed the authors to quote a part of his unpublished results. Exovates themselves cannot be fertilized, as Just ('23) previously showed. When the main body of the egg cell receives a spermatozoon and the fertilization membrane is formed around it, the exovate sometimes forms a very thin membrane. But on other occasions, it fails to form it entirely so that when it cleaves, after migration of nuclei into it, the blastomeres fall apart. This fact is considered to indicate that the cortical layer is absent on exovates; or if it exists, it must be thinned out greatly because of stretching. On the basis of such an explanation, if the cortical birefringence is caused merely by the quantity of cortical substance present, the birefringence of the exovate should be very weak, since the cortex there must be thinner than elsewhere. But the facts indicate the contrary. This means that besides the quantity of the cortical substance, there must be another factor involved which plays a predominant role. The authors believe this to be a tension factor. This point will be taken up once more in section (9).

(4) When unfertilized eggs of *Pseudocentrotus* are caused to elongate by centrifugation, a notable difference in the cortical condition from previous cases is that the surface of the centrifuged eggs acquires a stronger negative birefringence uniformly over the entire cell. The obvious explanation here

is that the gradual stretching by centrifugation allows ample time for regional differences in tension to be equalized.

When an egg is sucked into a capillary and deformed, there has been no way to prove whether the cortex is stretched uniformly over the entire surface or only in the cylindrical part, or whether, perhaps, the cortex is held back toward the hind end as a result of adhesiveness between the egg surface and the capillary wall. In this connection, the facts observed in (1), (2), (3) and (4) are quite instructive in indicating which part of the egg surface is being stretched, and that such regional differences are equalized on standing. *Astriclypeus* eggs are particularly interesting in this connection.

(5) In the eggs of the sand dollar, *Astriclypeus manni*, the cortical birefringence is almost imperceptible. Consequently, only the stretched parts are shown up by local birefringence when the capillary or exovate experiment is performed.

(6) Unfertilized eggs of *Pseudocentrotus* and *Heliocidaris*, observed while they are swelling in a hypotonic medium, show a marked increase in cortical birefringence. There might be some danger in drawing such a conclusion if only the bright quadrants are examined, because of the increased brightness acquired by the egg cytoplasm as it is diluted. Fortunately, however, since a mica plate is being used, the observation can be checked by watching the dark quadrants. Here, contrary to the increase in brightness of the cytoplasm, the cortex becomes darker as its birefringence gets stronger.

(7) Among many fertilized eggs of *Pseudocentrotus*, there are a few cases in which a part of the cytoplasm is exuding through a small hole in the fertilization membrane. In such a pinched egg, the part exuding from the membrane invariably has a stronger birefringence than the part left within the fertilization membrane.

(8) Finally, fertilized eggs of *Pseudocentrotus*, exposed to the same hypertonic medium as that used in the exovate experiment, were observed with the polarization microscope. In the shrunken eggs, the birefringence of the cortical layer

reverses its sign and becomes positive in the direction of the tangent.

(9) The report so far has been concerned with birefringence in the eggs of the sea-urchins, *Pseudocentrotus* and *Heliocidaris*, and of the sand dollar, *Astriclypeus*, all of which change similarly under similar conditions. On the contrary, the results obtained with the eggs of the sea-urchin, *Mespilia globulus*, turned out to be diametrically opposite. This is a fact of extreme importance. The unfertilized eggs of this species have a fairly strong cortical birefringence. When they are stretched by being drawn into a capillary, the birefringence becomes generally weaker. But the cylindrical part which is believed to be stretched (and hence acquires stronger birefringence in other forms) now has a weaker birefringence than the round end parts. Eggs examined in the capillary sometimes show the front end shining more brightly than the hind end, as is the case with other forms, but just as frequently, the reverse situation is obtained.

If the unfertilized *Mespilia* eggs are treated with a hypotonic (60%) and a hypertonic (120–150% by adding NaCl) medium and their birefringence compared with that in normal sea water, the former is much weaker and the latter is decidedly stronger than normal, which is exactly opposite to the results obtained with other eggs. Moreover, the birefringent layer is obviously thinner in the hypotonic and thicker in the hypertonic medium than the control.

The above results may indicate that the stretch of the cortical birefringence of *Mespilia* eggs is proportional to the amount of cortical substance. As was discussed in (3), in the double refraction of a gel, two separate factors are involved —a tension factor and a quantity factor. Therefore, if the assumption is allowed that in *Mespilia* the quantity factor overcomes the tension factor, at least a tentative explanation is provided for the *Mespilia* results.

Strictly speaking, the "tension factor" is the expression of an increase in regularity of arrangement of the component micelles in a given structure as the result of tension, but it is

possible to imagine a case in which the application of tension would fail to produce this result. There is a variety of evidence indicating that the cortical layer of *Mespilia* eggs is a much softer gel than that of the other eggs studied: they cytolize and fragment unless handled with special care, and often cytolize spontaneously on standing for a few hours in sea water. The fact that these eggs have an exceptionally thick hyaline layer (Dan, Yanagita and Sugiyama, '37) may also point to an extreme lability of the cortex.

(10) The fertilization membrane of *Mesphilia* eggs has an unusually weak birefringence positive in respect to its tangent. Since this situation is so different from other cases in which the birefringence of the fertilization membrane is stronger than that of any other cell structure, it was investigated by adding egg albumin to the egg suspension in order to reduce the turgor of the fertilization membrane. As the membrane became crenated and then rounded up, much reduced in size, its luminosity became progressively weaker, and was finally almost imperceptible. This is taken as evidence that the tension factor is responsible for the birefringence of the *Mespilia* membrane.

On the whole, the results so far collected empirically support the authors' argument that when a doubly refractive gel is put under tension, the magnitude of its original birefringence increases; and when it is compressed, the magnitude decreases towards zero, and in some cases even a birefringence of the opposite sign develops. This idea, although it is rather crude in its present form, is quite useful in its practical application.

The endeavor of biologists who have been studying birefringence in recent years has been directed to the analysis of the cause of birefringence, down to the molecular orientation of basic substances such as proteins, lipids, nucleic acids, etc. Roughly speaking, their research falls into two main categories. One is the study of form birefringence, and the other is the study of intrinsic birefringence. Unfortunately, however, in spite of the accuracy of the knowledge accumulated,

the methods used are not directly applicable to living cells. The imbibition method, which is ordinarily used for the study of form birefringence, requires the immersion of materials in liquids of various refractive indices; while the solution method, for the analysis of intrinsic birefringence, necessitates treatment with various solvents specific for one component or another.

As long as these orthodox methods cannot be used for living materials, it becomes necessary to resort to methods which, although cruder, do fulfill the primary condition of maintaining vitality. The idea that tension changes may be at the root of birefringence changes is not a new one, but in the field of biology, it has hitherto been looked upon as a mere suggestion.

However, so far as transient changes in birefringence of living structures such as those here reported are concerned, to assume that the basic substances of the structures do not change within a few minutes and that tension plays a predominant role will be far less provocative than to put materials through drastic killing processes and imagine living conditions from dead figures. The present paper points out the necessity for further pursuit along this line of attack.

We express our thanks to the staff of the Misaki Marine Biological Station for placing their facilities at our disposal. The present research was supported by a grant from the National Research Council of Japan for 1948 for which the authors are happy to express their gratitude.

SUMMARY

Echinoderm eggs were deformed by sucking them into glass capillaries, and the change of birefringence of the cortical layers studied. In most of the eggs the tangentially negative birefringence increased whenever the cortex was pulled, while it decreased where the cortex was compressed.

The birefringence change in the cortex of the egg of *Mespilia globulus,* however, was exactly the opposite. This differ-

ence is explained by assuming that birefringence is a function of both tension and the quantity of material, the latter factor being greater in the *Mespilia* egg cortex.

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THE RELATIONSHIP OF THE CELL SURFACE TO METABOLISM

VII. THE KINETICS AND TEMPERATURE CHARACTERISTICS OF URANIUM-INHIBITION [1]

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FOUR FIGURES

In previous papers of this series (Rothstein and Larrabee, '48; Rothstein, Frenkel and Larrabee, '48; Rothstein, Meier and Hurwitz, '51; Rothstein and Meier, '51), it has been shown that the uranyl ion specifically inhibits the metabolism of hexoses by forming a highly undissociated but reversible complex with specific loci on the surface of the cell. These loci possess certain chemical characteristics typical of polyphosphates, suggesting that uranium interferes with sugar metabolism by preventing phosphorylation reactions on the surface of the cell. This hypothesis is predicated on the concept that sugars are actively transported into the cell by a mechanism involving enzyme reactions on the cell surface, with consequent chemical alteration of the sugar molecule as it passes through the cell-membrane.

Assuming that the above hypothesis is essentially correct, then the uranium-inhibited reactions are enzymatic reactions and should display the general properties of enzymatic reactions. An alternative hypothesis is based on the classical concept of permeability, in which the cell-membrane

[1] This paper is based on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, New York.

[2] Part of the data in this paper was taken from a thesis presented by Leon Hurwitz in partial fulfillment of the requirements for the degree of Master of Science, University of Rochester, 1950.

regulates the rate of diffusion of substrate through it into the cell interior, by its properties as a semi-permeable membrane. For example, Barron et al. ('48) suggested that uranium inhibits glucose metabolism by combining with proteins of the cell-membrane, thereby reducing its permeability to the substrate. There is a fundamental difference in the two hypotheses which may be investigated experimentally. In the first hypothesis, the rate-limiting factor in the presence of inhibiting concentrations of uranium is an enzyme reaction, whereas in the second, it is a diffusion phenomenon or some property associated with permeability.

In the present paper a study has been made of the kinetic properties and of the temperature characteristics of the uranium-inhibition of fermentation and of respiration of living yeast cells. The data obtained are shown to be most consistent with the hypothesis that the uranium-inhibited reactions are enzymatic in nature.

METHODS

The methods of treating and handling the yeast cells are the same as those described in the previous papers of this series. Briefly, fresh commercial Baker's yeast (Standard Brands) was thoroughly washed, then starved with aeration for one to three hours before use. Rates of metabolism were determined with a standard Warburg apparatus. In all experiments with uranium, the uranyl nitrate solution was tipped into the yeast prior to the glucose in order to avoid the lag period in the appearance of inhibition (Rothstein, Meier and Hurwitz, '51). The pH's of all solutions and of the yeast suspension were adjusted to 3.5 before the start of each experiment. Uranium analyses were done by isotope technique (Rothstein, Frenkel and Larrabee, '48), using samples of natural uranium enriched with U233.

RESULTS

The influence of substrate concentration on the rate of nearly all enzyme reactions conforms to the Michaelis-Menten

equation or its modifications (Lineweaver and Burk, '34). This equation is predicated on the existence of a substrate-enzyme complex, whose concentration determines the overall rate of the reaction. Thus

$$E + S \rightleftharpoons ES \rightarrow \text{product} \qquad (1)$$

where E and S are enzyme and substrate concentrations, and ES is the concentration of enzyme-substrate complex. From

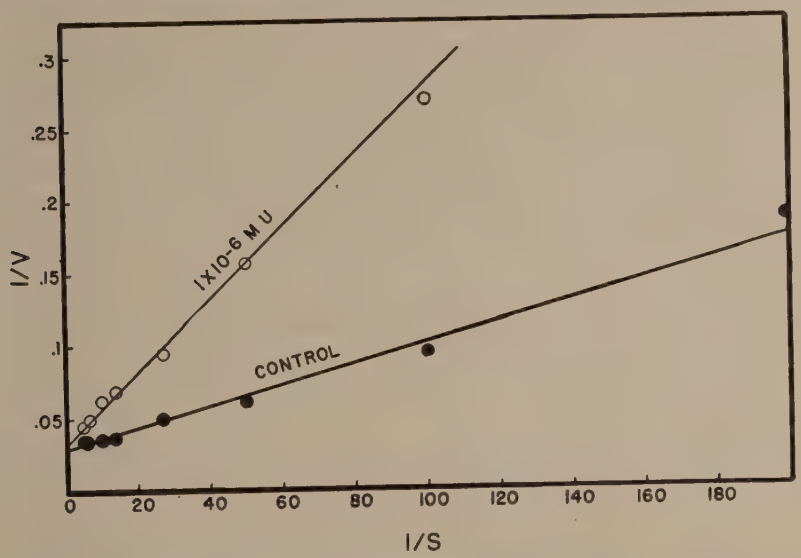

Fig. 1 Kinetics of inhibition of fermentation of fructose.
Substrate concentrations (S) are expressed in M/l. Rates (V) are in μl of CO_2 per mg of yeast (wet wt.) per hour. The yeast concentration was 4.0 mg/ml, the temperature, 25°C., and the pH, 3.5.

mass law considerations the following equation can be obtained:

$$1/V = Km/Vm \cdot 1/S + 1/Vm \qquad (2)$$

where V is the rate of the reaction, S, the substrate concentration, Vm, the theoretical maximal rate of metabolism, and Km, the Michaelis constant. According to this equation a plot of 1/V against 1/S should be a straight line. In figures 1, 2 and 3 such plots of 1/V against 1/S have been made for representative data for glucose fermentation and respiration,

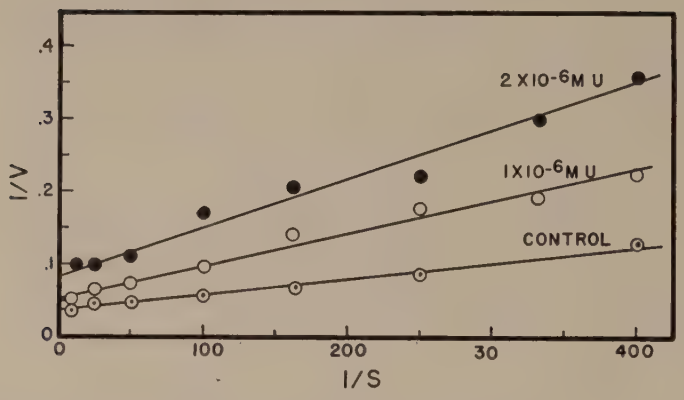

Fig. 2 Kinetics of inhibition of fermentation of glucose.

Substrate concentrations (S) are expressed in M/l. Rates (V) are in μl of CO_2 per mg yeast (wet wt.) per hour. The yeast concentration was 5.15 mg/ml, the temperature 25°C., and the pH 3.5.

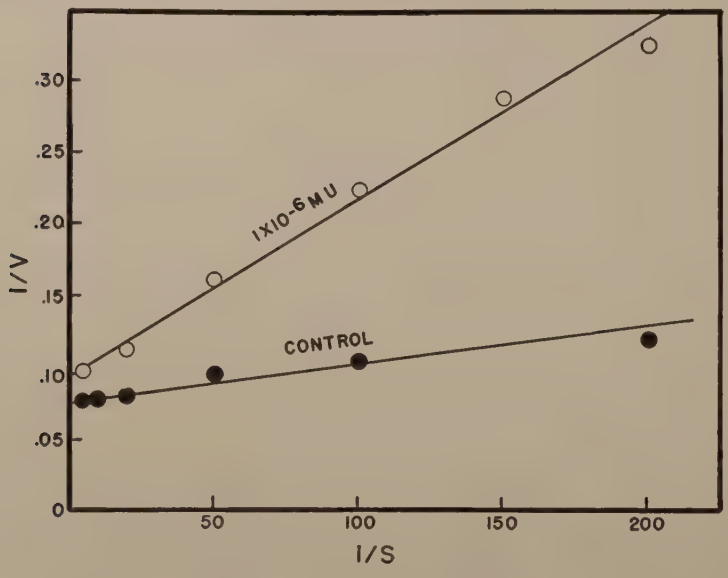

Fig. 3 Kinetics of inhibition of respiration of glucose.

Substrate concentrations (S) are expressed in M/l. Rates (V) are in μl of O_2 per mg yeast (wet wt.) per hour. The yeast concentration was 6.4 mg/ml, the temperature, 25°C., and the pH, 3.5.

and for fructose fermentation, in the presence and absence of uranyl nitrate. In each case, the data could be fitted reasonably well by a straight line. The values for Vm calculated from the intercept, and for Km calculated from the slope, are listed in table 1.

In the case of the control data the values for Vm for fructose and for glucose fermentation were approximately the same, about 30 µl/hr/mg. However the Km for glucose was considerably lower than that for fructose, 7.2×10^{-3} as compared to 2.9×10^{-2}. These results are in essential agreement with those previously obtained by Hopkins and Roberts ('35)

TABLE 1

Summary of kinetic data from figures 1, 2 and 3

SUBSTRATE	MODE OF METABOLISM	UO_2++ CONC.	Vm	SLOPE	Km CALC. FROM EQUATION (1)
		M/1	*µ/hr/mg*		
Fructose	fermentation	0	32	9.0×10^{-4}	2.9×10^{-2}
		1×10^{-6}	31	2.5×10^{-3}	7.8×10^{-2}
Glucose	fermentation	0	30	2.4×10^{-4}	7.2×10^{-3}
		1×10^{-6}	19	4.3×10^{-4}	8.2×10^{-3}
		2×10^{-6}	12	6.9×10^{-4}	8.3×10^{-3}
Glucose	respiration	0	14	2.7×10^{-4}	3.8×10^{-3}
		1×10^{-6}	11	1.7×10^{-3}	1.9×10^{-2}

and Gottschalk ('44). The values of Km for respiration of glucose were not much different from those for fermentation — 3.8×10^{-3} as compared with 7.2×10^{-3}. However, the Vm was considerably lower, 14, as compared with 30 µl/mg/hr.

As pointed out previously, the presence of an inhibiting concentration of uranium did not result in deviations from the Michaelis-Menten formulation. In each experiment, the reciprocal plot of 1/V against 1/S gave a straight line. In the case of fructose fermentation the intercept (Vm) was the same in the presence and absence of uranium, but the slope was higher with uranium (fig. 1). Consequently, the Km was higher (table 1), 7.8×10^{-2} compared to 2.9×10^{-2}. In other words, at low substrate concentrations, there was a marked

inhibition by uranium, but at the highest substrate concentrations the inhibition was completely reversed. This behavior is typical of a competitive inhibition (Lineweaver and Burk, '34). In the case of glucose fermentation, however, the results were different. There was a shift in both the slope *and* the intercept (fig. 2) typical of a non-competitive inhibition. In a purely non-competitive system, the Km should be the same in the presence and absence of inhibitor. In the case of glucose fermentation this was true within the limits of the experimental technique (table 1). The Km was 7.2×10^{-3} in the control, as compared to 8.2×10^{-3} in the presence of uranium.

At first the marked difference in the kinetics of inhibition of glucose as compared to fructose metabolism seemed puzzling, particularly in view of the fact they are handled similarly in the metabolic scheme. However, this apparent discrepancy was resolved when it was found in other studies that fructose forms a weak but definite complex with uranyl ion, whereas glucose does not (Rothstein and Meier, '51). The competitive effect found in the fructose data (fig. 1) was a result not of a competition of substrate and inhibitor for surface loci, but rather a competition between substrate and enzyme for inhibitor molecules. The formation of a complex between the substrate and the inhibitor masks the true inhibition-kinetics. This experiment illustrates the danger of a kinetic analysis of inhibition, unless it can be shown that no interaction occurs between the inhibitor and substrate.

In contrast to the non-competitive inhibition found for glucose fermentation, the respiration of glucose can be characterized as a mixed inhibition, partly competitive, and partly non-competitive. The value for Vm was lower in the presence of uranium (fig. 3 and table 1) indicating a non-competitive element, but the value of Km was also increased 4-fold from 3.8×10^{-3} to 1.9×10^{-2}, indicating a competitive element. In 6 other experiments performed under the same conditions as in figure 3, some variation was encountered in the values of Vm and Km, but in each case, there was a mixed inhibition,

partly competitive and partly non-competitive. The difference between the kinetics of inhibition of fermentation as compared with those for respiration are not unexpected in view of the previous finding (Rothstein, Meier and Hurwitz, '51) that the two modes of metabolism are somewhat differently inhibited by uranium. Many more uranium-sensitive cell-surface loci are involved in respiration than in fermentation.

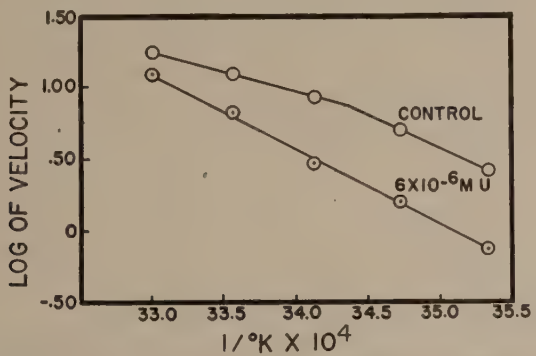

Fig. 4 Temperature characteristics of respiration of glucose in the presence and absence of uranium.

The velocity is expressed in μl O_2 per mg of yeast (wet wt.) per hour. °K is the temperature in degrees Kelvin. The yeast concentration was 6.5 mg/ml, the glucose concentration .2 M, and the pH, 3.5.

In addition to the kinetic characterization of uranium-inhibition, studies were made of the temperature characteristics in terms of the Arrhenius equation:

$$\log V = -2.3\,\mu/RT + K \qquad (3)$$

where V = rate of metabolism, μ the energy of activation, R the gas law constant, T the absolute temperature and K a constant. In figure 4 an Arrhenius plot has been made for the respiration of glucose in the presence and absence of an inhibiting concentration of uranium. The control data do not quite give a single straight line, but can be conveniently represented by a pair of lines with a "break" at a temperature of 18°C. The values for μ calculated from the two slopes were 21,000 cal/mol for the lower segment and 13,000 cal/mol

for the upper. These values are in essential agreement with those previously obtained by Stier, '33. In the presence of an inhibiting concentration of uranyl nitrate, a single straight line was obtained, with no indication of any "break." The value for μ was 23,000 cal/mol, approximately the same as that for the lower leg of the control curve. It is of interest to note that the lines for the control and experimental tended to converge at higher temperatures. In other words the inhibition was markedly decreased as the temperature was elevated, particularly at temperatures above the "break" in the control curve. For example, elevation of the temperature from 20° to 30°C. decreased the percentage inhibition from 66 to 29%.

TABLE 2

Uptake of uranium by cells as a function of temperature

The yeast concentration was 10 mg/ml, the pH, 3.5, and the initial uranium concentration, 5.0×10^{-6} M. The yeast and uranium were left in contact for 10 minutes, then the cells were separated out by centrifugation.

TEMP.	FINAL U CONC. OF SUPERNATE	% OF U TAKEN UP BY CELLS
°C.	*M/1*	
15	1.37×10^{-6}	97.3
20	1.36×10^{-6}	97.3
25	1.02×10^{-6}	98.0
30	1.38×10^{-6}	97.2

Some data were also obtained concerning temperature characteristics for glucose fermentation. These data were about comparable to those for respiration. In the presence of uranium the value for μ was again of the order of 22,000 cal/mol.

The temperature characteristics of 22,000 cal/mol, shown in figure 4, might be a property of the rate-limiting metabolic system, or might result from an increased dissociation of the uranium-cell surface complex at higher temperatures, or a combination of both. However, in table 2, it is shown that under conditions similar to those used in the experiments of

figure 4, changes in temperature from 15° to 30°C. do not appreciably alter the uptake of uranium by the cells. Thus the temperature characteristic of figure 4 must be characteristic of the inhibited metabolic step.

DISCUSSION

Because uranyl ion acts on the surface of the cell (Rothstein and Larrabee, '48) this ion undoubtedly inhibits glucose metabolism by interfering in some manner with the passage of glucose through the cell membrane. Consequently the kinetics and temperature characteristic of glucose metabolism in the presence of an inhibiting concentration of uranium must necessarily represent characteristics of surface reactions and may serve to identify the nature of the phenomena involved. Thus, if the passage of glucose through the membrane involves enzymatic alteration of the glucose molecule, then the kinetics and temperature characteristics should be consistent with those expected of enzyme reactions. On the other hand, if passage of glucose through the membrane involves a simple diffusion phenomenon, the kinetics and temperature characteristics should be consistent with those expected of such diffusion phenomena.

Enzyme reactions in general can be characterized by the Michaelis-Menten equation or one of its modifications. Inherent in this equation is the concept of the formation of a substrate-enzyme complex and the presence of a maximal rate of metabolism associated with the saturation of the available enzyme by an excess of substrate. On the other hand, diffusion phenomena can be characterized by the Fick equation or its modifications in which the rate of diffusion for a substance is proportional to its activity gradient. In the present case, if the diffusion of glucose across the cell-membrane is the rate-limiting process in metabolism (as it would be if the observed inhibition by uranium were due to interference with the diffusion of glucose into the cell), the glucose would be metabolized almost as fast as it reached the interior of the cell. The glucose concentration inside the cell would conse-

quently approach a low value, and the activity gradient across the membrane would be essentially equal to the external concentration of glucose. Under these conditions, the rate of metabolism should also be essentially proportional to the external glucose concentration, with little saturation effect and no asymptotic relationship between substrate concentration and the rate of metabolism. Because it has already been shown that the kinetics of glucose metabolism in the presence of uranium are consistent with the Michaelis-Menten equation, the data are inconsistent with the hypothesis that uranium interferes with the simple diffusion of glucose through the cell membrane.

In the case of the temperature characteristic, the value for the energy of activation μ in the presence of uranium was 22,000 cal/mol, a value indicative of a reaction involving a major energy shift. Such a value is consistent with the enzyme hypothesis (Sizer, '43) but is inconsistent with diffusion phenomena, which have low energies of activation, of the order of 3000–5000 cal/mol (Glasstone, Laidler and Eyring, '41).

Although it seems evident from the above discussion that glucose does not pass into the cell by simple diffusion, there are other phenomena often considered to be important in permeability which should be considered. These are lipid solubility and adsorption-desorption. The importance of lipid solubility as a factor in permeability has long been recognized. Those substances which have a high partition coefficient between oil and water, tend to penetrate the cell membrane much more rapidly, undoubtedly by actually dissolving in the lipid phase of the cell-membrane. In the case of glucose, with its extremely low lipid solubility, this factor would seem to be unimportant. However, adsorption cannot so readily be discarded as an important factor particularly if it is broadly defined to include the formation and dissociation of complexes. Certainly in the case of ions passing through the cell membrane such interactions undoubtedly play an important role. For example, in the case of sodium, it has been

postulated that this ion is actively transported by a mechanism involving the formation of a complex which then dissociates the ion at the inner face of the membrane (Ussing, '49). Obviously such adsorption mechanisms differ fundamentally from enzyme reactions, since dissociation of the enzyme-substrate complex yields a product, chemically distinct from the substrate.

Adsorption-desorption phenomena, if rate-determining in metabolism, could result in kinetics consistent with the Michaelis-Menten equation, and conceivably, depending on the exact nature of the adsorption-desorption process, could give values for μ, in the range of those typical of enzyme reactions. Thus kinetics alone can not necessarily differentiate between enzyme reactions and adsorption phenomena. However other available information, reviewed below only briefly, would seem to be less consistent with an adsorption hypothesis than with an enzyme hypothesis.

Firstly, it was found that the surface loci involved in fermentation are not the same as those involved in the respiration of glucose (Rothstein, Meier and Hurwitz, '51). The existence of at least two different kinds of surface-loci is reflected in the qualitative differences in kinetics between aerobic and anaerobic metabolism shown in this paper. Secondly, galactose metabolism of adapted cells is more sensitive to inhibition by uranium than is glucose metabolism (Rothstein, Meier and Hurwitz, '51), indicating that different surface loci are involved in the metabolism of the two sugars. Thirdly, uranyl ion has a remarkable capacity to form relatively undissociated complexes with various anions as it also does with the cell-surface groups. Chemically, the cell-surface groups behave toward uranyl ion as do the polyphosphates, whereas other anions of biological importance behave differently. This has led to the conclusion that the cell-surface groups are chemically related to the polyphosphates (Rothstein and Meier, '51).

Although to the knowledge of the present authors, no specific mechanism of glucose uptake by an adsorption-desorp-

tion mechanism has been postulated, such a mechanism cannot automatically be excluded for this reason. However, an adsorption hypothesis would have to be quite complicated to take into account the aerobic-anaerobic differences, the substrate specificity, and the chemical nature of the surface loci. There would have to exist at least two kinds of adsorption mechanisms, one operative under aerobic and one under anaerobic conditions. There would have to be different adsorption sites for glucose and for galactose. The adsorption hypothesis would have to take into account the chemical nature of the surface loci. Neither uranyl ion nor polyphosphates form complexes with glucose. It is difficult to see how polyphosphates could be involved in any adsorption or complex formation with glucose, or how uranyl ion could interfere with glucose adsorption.

On the other hand, the enzyme hypothesis can readily take the facts into account. Anaerobic-aerobic differences as already pointed out could be attributed to a divergence in enzyme pathways under the two sets of conditions (Rothstein, Meier and Hurwitz, '50). Substrate specificity in the case of galactose and glucose is consistent with the fact that different enzymes (kinases) are involved for each of these substrates (Trucco, '48). The polyphosphates and phosphorylations reactions are known to be intimately involved in the enzyme reactions of sugar metabolism.

SUMMARY AND CONCLUSIONS

1. The relationship between substrate concentration and the rate of fermentation and respiration of glucose and fructose could be fitted by the Michaelis-Menten equation in the presence or absence of an inhibiting concentration or uranium as uranyl ion.

2. In the case of fructose fermentation the kinetics were typical of a competitive inhibition. This was shown to be a result of complex formation between uranyl ion and fructose, rather than a competition between inhibition and substrate for the enzyme.

3. Glucose which does not complex with uranyl ion showed kinetics of inhibition of fermentation which were typically non-competitive. On the other hand, the kinetics of inhibition of respiration were more complicated, involving both non-competitive and competitive elements.

4. The temperature characteristic for glucose fermentation was higher (22,000 cal/mol) in the presence of uranium than in its absence. Thus elevation of the temperature caused reversal of inhibition. This was not due to a decreased uranium-uptake by the cells.

5. It was concluded that the kinetic data and temperature characteristic were incompatible with the concept that glucose enters the cell by simple diffusion.

6. Other mechanisms of permeability were discussed including adsorption-desorption phenomena defined broadly to include the formation and dissociation of complexes. It was suggested that although such mechanisms could explain some of the kinetic data, they were not compatible with other available information.

7. It was suggested that the best hypothesis to explain the existing information, involved the chemical alteration of sugars by enzymes located on the surface of the cell.

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PHOSPHATASE IN THE ADULT WORKER HONEY BEE[1]

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FIVE FIGURES

The importance of the phosphatases, specifically the monophosphoesterases, especially in nucleic acid and carbohydrate metabolism, has suggested the possibility that such enzymes might play an important role in the physiology of aging of animals. However, as in the case of most enzymes studied intensively in higher animals, little if any work has been done on the occurrence and chemistry of phosphatase, in insects in general, and in the adult honey bee, in particular.

Near the end of the present study, there appeared a report by Fitzgerald ('49), concerning alkaline phosphatase in grasshopper eggs, which also included a review of most of the (limited) relevant literature up to 1948. Since that time there were published a later review on alkaline phosphatase by Roche and Thoai ('50), on the phosphatases by Roche ('51), as well as several reports on the occurrence of phosphatase in insects (chiefly histochemical studies) by Doyle ('48), Day ('49), and Hébert ('50). Other than an earlier report by Busnel and Drilhon ('45, '45a) that of Fitzgerald is one of the few thorough biochemical studies on this enzyme in insects.

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[2] Research assistant.

[3] The authors are indebted to Mr. Leonard Levine for his invaluable technical assistance in the completion of the final set of determinations, as well as for his assistance with the line drawings.

[4] Contribution from the Laboratories of Zoophysiology.

The present study was undertaken to determine the chemistry of and the optimal conditions for the study of β-glycerophosphatase in the adult worker honey bee, *Apis mellifera* L.

METHODS

Homogenizing procedure

A known number of bees were decapitated and the weight of the complete animals (heads and bodies together) determined. These were then homogenized in lots of 20 bees per 15 ml of cold distilled water, in an ice-packed semi-micro Waring Blendor, in a cold room (5°C.). The homogenate was filtered under vacuum through 8 gm of Pyrex 800 glass wool, evenly spread across a 100 mm Büchner funnel, into a 600 ml filter flask. Homogenizer cup, funnel and flask were each washed quantitatively with cold distilled water into a 50 ml volumetric flask and final dilution made to mark with distilled water. Thus each 5 ml of the final, diluted homogenate contained two complete bees (average weight 0.1 gm per bee). The advantages of the total homogenate method have been stressed elsewhere (Potter, '47; Moog, '46, '46a; Rockstein, '50).

Ten-ml aliquots of homogenate were transferred into shell vials each containing a drop of chloroform and fast frozen in a freezer (— 25°C.), where they were stored until needed.

General enzyme procedure [5]

Vials were removed and allowed to thaw at room temperature (one hour), the contents shaken thoroughly, and 3-ml aliquots of homogenate (each containing one and one-fifth bees) transferred to 20-ml glass-stoppered incubation tubes. To each incubation tube were then added 4 ml of sodium barbital-buffered sodium β-glycerophosphate solution (see below) and 1 ml of 0.8 M $MgCl_2$.[6] After the addition of a drop of

[5] As indicated below, each of the several conditions of the procedure was varied depending on the particular experimental parameter under study.

[6] Determined by preliminary study employing varying concentrations of $MgCl_2$. (See *Miscellaneous data*, below.)

chloroform, the tubes were stoppered, mixed by shaking, and incubated in a water bath maintained at 37°C., for a period of 5 hours. The enzyme action was stopped by placing incubation tubes into a boiling water bath for 5 minutes and then cooling under cool tap water. One ml of 30% trichloroacetic acid was added to the cooled incubation mixture and after a 5-minute wait, the precipitated proteins were filtered through 9-cm Whatman 44 filter paper. A control tube was prepared whose contents and treatment were identical with each experimental tube, except that the control tube was subjected to the boiling water bath *before* incubation.

Determination of activity

Enzyme activity was measured by the authors' adaptation (Rockstein and Herron, '51) of Sumner's ('44) ferrous sulfate-acid molybdate method for the colorimetric determination of released phosphate as phosphorus, which method employs 0.2-ml samples of filtrate or standard phosphate solution, plus 9 ml of acid molybdate plus 0.8 ml of 10% $FeSO_4$. Measurements were made in matched plastic cells in the Model B Beckman spectrophotometer. All determinations, as indicated by mean values plotted in the figures (below) were made in quintuplicate, with one stated exception. In each study, replications were made from a single large homogenate sample.

RESULTS[1]

Enzyme concentration

Figure 1 shows the relationship between enzyme activity, expressed as μg P released per 0.2 ml of filtrate sample, and enzyme concentration expressed as ml of homogenate. These data represent average values from 5 sets of replications made from two different homogenate samples of bees

[1] Final activity was calculated after subtracting absorbance of control tube from that of experimental tube and determining P released enzymatically from absorbance value of standard phosphate solution. Activity in all graphs is expressed as μg P released per 0.2 ml.

of less than 24 hours old. The straight line portion of the curve was drawn by the method of least squares for all points except that for 3.0 ml, which represents no increase over the previous point. The 4 ml of substrate used consisted of 0.5% (0.016 M) sodium β-glycerophosphate·$5H_2O$ (Eastman Kodak; maximum α 0.1%), buffered by 0.424% sodium diethylbarbiturate (barbital) (Merck); Bodansky, '33). These concentrations of substrate and buffer are called

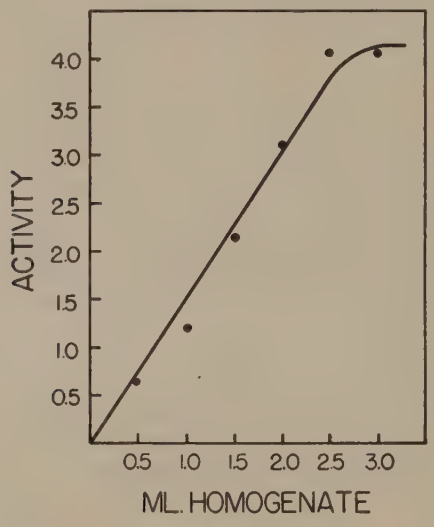

Fig. 1 Alkaline phosphatase activity and enzyme concentration; adult worker honey bee. Variable homogenate; 4 ml of *normal* glycerophosphate-*normal* barbital; 1 ml 0.8 M $MgCl_2$; pH ~ 8.3. Incubation time: 5 hours at 37°C. Age of bees: less than 24 hours old.

normal throughout this report for purposes of identification. All samples of homogenate less than 3 ml were diluted to 3 ml total with distilled water, where necessary, to bring the final incubating solution to a total of 8 ml, including the substrate and 1 ml of 0.8 $MgCl_2$. For all studies following this one, 3 ml of homogenate, containing a total of one and one-fifth complete bees, was decided upon as the enzyme sample to be employed.

Substrate concentration

Figure 2 demonstrates the effect upon enzyme activity of increasing concentrations of the substrate, expressed as milliliters of substrate; concentration employed was 4 times *normal* substrate, *normal* barbital concentration. Volumes of this substrate under 4 ml were made up to 4 ml with *normal*

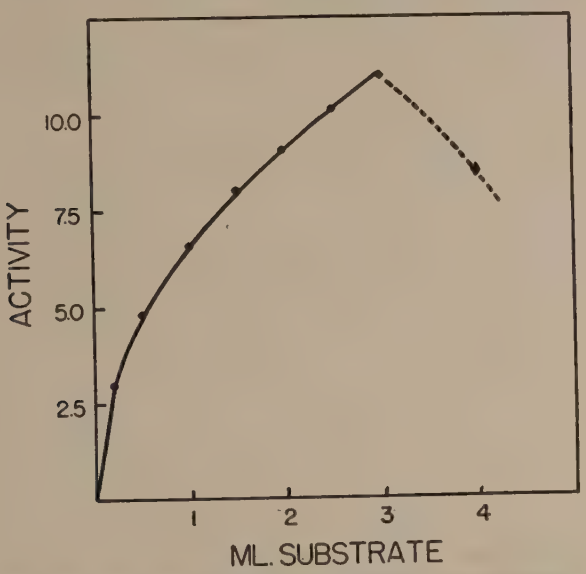

Fig. 2 Alkaline phosphatase activity and substrate concentration; adult worker honey bee. Three ml homogenate; variable 4 × *normal* glycerophosphate-*normal* barbital; 1 ml 0.8 M $MgCl_2$; pH ~ 8.1. Incubation time: 5 hours at 37°C. Age of bees: less than 24 hours old.

barbital, to bring the volume of the final incubating solution to 8 ml with 3 ml homogenate and 1 ml $MgCl_2$. Activity is seen to rise rapidly at very low concentrations of substrate, rising slowly thereafter and then falling sharply, according to the broken line portion of the curve (drawn for a single determination of 4 ml of substrate, indicated by the diamond symbol in fig. 2). (All other points in this figure represent mean values for 4 replications.) This decline is typical of several enzymes as reported by Van Slyke ('42), Sumner and

Somers ('47), and by Fitzgerald ('49) for insect phosphatase. The indicated optimum, final substrate concentration in the 8 ml of incubating solution for 3 ml of 4 × *normal* substrate was 0.024 M.

Varying pH

Figure 3 shows the effect of changing pH by the addition of 1 ml of varying concentrations of acetic acid or sodium hydroxide to 3 ml of 4 × *normal* glycerophosphate-2 × *normal*[8] barbital, 3 ml of homogenate and 1 ml of 0.8 M $MgCl_2$. Electrometric measurements of pH values showed a variation of less than 0.1 pH before and after the 5-hour incubation period (see table 1). The diamond symbol at pH 7.55 represents a single determination made to establish the curvature of the drawn curve in what was considered by the authors

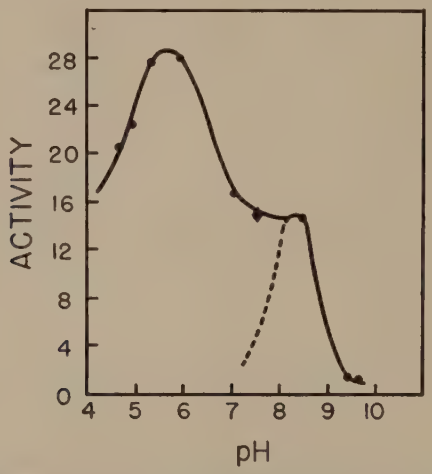

Fig. 3 Phosphatase activity and pH; adult worker honey bee. Three ml homogenate; 3 ml. 4 × *normal* glycerophosphate-2 × *normal* barbital: 1 ml 0.8 M $MgCl_2$; variable AcOH or NaOH, variable pH. Incubation time: 5 hours at 37°C. Age of bees: less than 24 hours old.

[8] It was found desirable to employ 2 × *normal* barbital in the pH, and in later *acid* phosphatase studies, to maintain buffering capacity for the incubation period employed, at low pH values especially.

to be a critical region. There are distinguishable a typical acid phosphatase activity curve with a pronounced maximum in the region of pH 5.6, and a less pronounced alkaline phosphatase curve with a less definite maximum suggested in the vicinity of pH 8.4. The broken line portion of the curve is drawn to suggest the possible activity curve of the alkaline phosphatase in the lower pH range of this enzyme, under the assumption that the more active acid phosphatase is masking the alkaline enzyme in the region indicated.

TABLE 1

The pH of incubating solution,[1] following addition of acid or base

ACID OR BASE ADDED	pH
0.5 ml 1N AcOH	4.60
0.4 ml 1N AcOH	4.88
0.3 ml 1N AcOH	5.26
0.2 ml 1N AcOH	5.88
0.1 ml 1N AcOH	7.04
0.05 ml 1N AcOH	7.55
1.0 ml H_2O	8.44
0.1 ml 1N NaOH	9.37
0.2 ml [2] 1N NaOH	9.62

[1] Three ml of homogenate, 3 ml of 4 × *normal glycerophosphate*-2 × *normal* barbital, 1 ml 0.8 M $MgCl_2$, and distilled water, where necessary, to 8 ml of total volume.

[2] Higher concentrations of NaOH produced a flocculent precipitate.

Kinetics of alkaline phosphatase

Figure 4 shows the change in activity as *total* phosphorus released at specific time intervals up to a maximum of 15 hours. The curve does not indicate a zero order reaction (activity/time is not a constant), except within the first few hours of the reaction under the conditions indicated under figure 4. Analysis for a first order reaction also failed to reveal a constant relation for log a/a-x (a being the original substrate, x the substrate released at times t_1, t_2 . . . . t_{15}). After the completion of the alkaline phosphatase kinetics study,

two series of time activity determinations were made at a pH of *(ca.)* 5.2 to determine the straight-line (zero-order) portion of the time-study curve for acid phosphatase. Each series was studied under conditions identical to those of the pH study, with homogenate from two different lots of bees

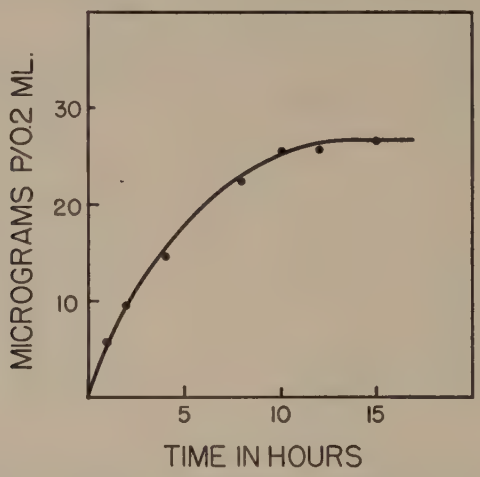

Fig. 4 Kinetics of alkaline phosphatase; adult worker honey bee. Three ml homogenate; 4 ml 3 × *normal* glycerophosphate-*normal* barbital; 1 ml 0.8 M $MgCl_2$; pH ~ 8.1. Incubation time variable at 37°C. Bees were "old" bees of unknown age.

less than 24 hours old. The following results indicate a similar absence of a zero order reaction *after* the first three hours:

TIME IN HOURS	ACTIVITY IN μG P/0.2 ML	
	series 1	*series 2*
1½	8.9	6.4
3	17.0	13.6
6	28.2	23.7

Each activity value represents the average of two readings for each time interval indicated.

Temperature and enzyme activity

Figure 5 shows the effect of varying temperature on alkaline phosphatase activity. This is a typical, inverted U-

shaped temperature activity curve with a steeper slope in the right side of the curve; i.e. at higher temperatures. Optimum temperature for the conditions described for this enzyme study lies in the region of 35°. The energy of activation (critical thermal increment), calculated graphically between 5° and 35°, was found to be 11,300 cal./mole. Q_{10} values were calculated for the same range of 5° to 35° as follows:

Temperature range	Q_{10}
5–15	2.38
15–25	1.78
25–35	1.38

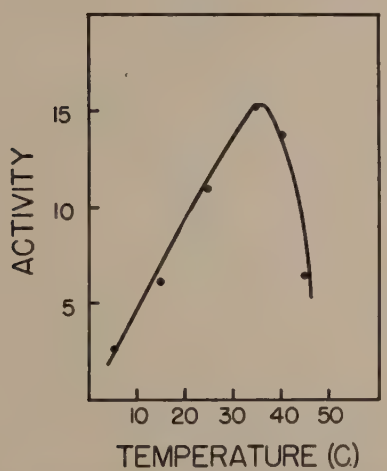

Fig. 5 The effect of temperature on alkaline phosphatase activity; adult worker honey bee. Three ml homogenate; 4 ml 3 × *normal* glycerophosphate-*normal* barbital; 1 ml. 0.8 M $MgCl_2$; pH ~ 8.1. Incubation time: 5 hours at 37°C. Bees were "old" bees of unknown age.

Miscellaneous data

The effect of magnesium upon alkaline and acid enzyme activity is shown in table 2, under the particular conditions of study indicated for each. The first set of data represents an early study, made to establish a general range within which to carry on future experiments; hence the conditions at considerable variance from those later employed after optimal

conditions of substrate and enzyme concentration had been determined. It is significant that in all cases indicated the presence of $MgCl_2$ resulted in an increase of up to 200% of original activity, depending on the several concentrations em-

TABLE 2

The effect of magnesium ions on phosphatase. Activity is expressed as µg/0.2 ml color reacting solution; $MgCl_2$ was added in 1-ml volumes of indicated concentrations

ENZYME	MG CL_2	ACTIVITY (µg P)
Alkaline [1]	0.000M	1.4
phosphatase	0.006M	1.4
	0.060M	1.8
	0.600M	2.5
Alkaline [2]	0.080M	2.9
phosphatase	0.200M	3.1
	0.400M	4.0
	0.800M	5.2
Acid [3]	0.008M	8.8
phosphatase	0.080M	18.4
	0.800M	14.7

[1] Conditions of this experiment included: 3 ml homogenate of bees less than 24 hours old; 2 ml *normal* substrate-*normal* barbital: pH undetermined; incubation temperature 37°C.; incubation time 4 hours. 0.000M $MgCl_2$ signifies 1 ml of distilled water. Activity represents value from a single determination.

[2] Conditions of this experiment included: 4 ml homogenate of "old" bees, age unknown; 4 ml 3 × *normal* substrate-*normal* barbital; pH ~ 8.1; incubation temperature 35°C.; incubation time two hours. Activity represents mean value from duplicate determinations.

[3] Conditions of this experiment included: 3 ml homogenate of bees less than 24 hours old; 3 ml 4 × *normal* substrate-2 × *normal* barbital; 1 ml 0.3 M AcOH; 1 ml of $MgCl_2$ of varying concentration; incubation temperature 35°C. incubation time 1½ hours. Activity represents mean value from duplicate determinations.

ployed. Activation of acid phosphatase by magnesium would definitely keep this enzyme out of class A2 of Folley and Kay's classification ('36, '37); (see DISCUSSION, below).

Table 3 shows the relative activity, employing identical conditions for the two substrates in each case, as indicated, of

alkaline and acid enzymes upon sodium β-glycerophosphate and disodium phenyl phosphate. In each case glycerophosphate hydrolysis was twice as rapid as that of phenyl phosphate.

TABLE 3

Comparative activity of acid and alkaline phosphatase for different substrates. Activity is expressed as μg P/0.2 ml

SUBSTRATE	ALKALINE ENZYME [1]		ACID ENZYME [2]	
	pH	Activity	pH	Activity
Sodium β-glycerophosphate	8.1	14.4	5.2	3.3
Disodium phenyl phosphate	8.0	6.2	5.1	1.3

[1] Conditions of this experiment included: 3 ml homogenate of bees less than 24 hours old; 4 ml 3 × *normal* substrate-*normal* barbital (final substrate concentration in 8 ml of incubating solutions — 0.024M); 1 ml 0.8M $MgCl_2$; incubation temperature 35°C.; incubation time 5 hours. Activity represents mean value from triplicate determinations.

[2] Conditions of this experiment included: 3 ml homogenate of bees less than 24 hours old; 3 ml 4 × *normal* substrate-2 × *normal* barbital (final substrate concentration in 8 ml of incubating solution — 0.024M); 1 ml 0.3M AcOH; 1 ml 0.8M $MgCl_2$; incubation temperature 35°C.; incubation time 1½ hours. Activity represents mean value from duplicate determinations.

DISCUSSION

Since the major interest of this study was a quantitative characterization of the phosphomonoesterase(s) of the honey bee, it should be noted that the alkaline enzyme suggested above falls into class A1 of Folley and Kay ('36, '37). The acid enzyme, showing activation by magnesium may be their type A4, but study on the relative hydrolysis on the α- and β-glycerophosphate, unfortunately not studied in this laboratory, would be necessary to confirm this. However, according to the Bamann-Meisenheimer classification ('41) based on pH of maximum activity, the two enzymes would fall into type 1 (pH optimum *ca.* 9) and type 3 (pH optimum *ca.* 5.5), respectively.

Although the two studies were based on completely dissimilar procedures of enzyme study, since no other really extensive quantitative study of phosphatases in insects has come to the attention of the authors, our findings on the alkaline enzyme of the honey bee are compared with those of the same enzyme in grasshopper eggs reported by Fitzgerald ('49), as follows:

	Adult honey bee	*Grasshopper eggs*
Optimum temperature	35°	30°
Optimum pH	8.4	8.5
Optimum Mg concentration	0.10 M	0.05 M

The optimum temperature reported here agrees with data of Lipman ('40) in marine invertebrates, as well as those of other workers on mammalian alkaline phosphatase (Bodansky, '39).

Differences in optimum magnesium concentration are of questionable significance, since as Roche ('46, '51), Moog ('46), Aebi ('49) and Fitzgerald ('49) have pointed out, pH differences, variation in enzyme concentration, and the presence of other co-activating or inhibiting factors often affect the "optimal magnesium concentration."

The kinetics data reported here agree with those of Fitzgerald ('49) as well as those for hydrolytic enzymes in general (Van Slyke, '42). Under the conditions of this study a zero order reaction is seen to obtain for at least two to three hours for both the acid and alkaline phosphatases. In this connection, Aebi ('49) showed that the time for a zero order reaction for alkaline kidney phosphatase hydrolysis of β-glycerophosphate could be varied by the presence or absence of magnesium, as well as by the particular buffer employed. Bodansky ('37) and Van Slyke ('42) have discussed at length the need for employing a time period for incubation which permits enzyme action to proceed at a maximal speed over the entire incubation period. As an alternative these workers suggested as more desirable the employment of the inverse time-enzyme relation (i.e. *the time necessary to obtain a fixed amount of hydrolysis)* to express enzyme activity. For de-

termination of enzyme activity by a colorimetric method like the one employed in the present study, this admittedly more desirable means of measurement of enzyme activity has definite limiting technical features. Any errors arising from such considerations can be vitiated by limiting the *time* of incubation to one to two hours for both enzymes under the conditions described for these enzymes.

The relatively high acid phosphatase activity, compared to the alkaline enzyme in the honey bee, may result from the methods of preparation of the enzyme, especially since alkaline phosphatase is reported as being salt soluble and acid enzyme as water soluble (Moog, '46a).

It is noteworthy that both acid and alkaline enzymes are twice as effective in hydrolyzing the glycerophosphate as the phenyl phosphate substrate, under identical conditions of study. Significance of this relative activity needs further clarification regarding the possible role of magnesium, pH as well as other experimental conditions of studying the enzymes.

The energy of activation reported here is slightly greater than that reported by Bodansky ('39) of 9,940 cal./mole both for cat and human bone phosphatase on sodium β-glycerophosphate, between 12° and 42°, at a pH of 9.0–9.2. In contrast to Bodansky's report of a constant value for the entire temperature range he employed, direct application of the Arrhenian equation to our data yielded values of 13,400 cal./mole between 5–15°, 10,500 for 15–25° and 5,900 for 25–35°. Although this apparently substantiates statements that enzyme systems do not follow the Arrhenian equation (Moelwyn-Hughes, '33; Sizer, '43; Bodansky, '39) has indicated the possible reason for this failure as being the method employed in expressing enzyme activity by workers in enzyme chemistry. This may apply to the present study. However, the Q_{10} values obtained, falling with an increase in temperature, show a general correspondence to those obtained in other enzyme studies (Sizer, '43).

In Lepidoptera, Drilhon and Busnel ('45, '45a) found strong acid phosphatase activity, with sodium β-glycerophosphate substrate, in the Malpighian tubules, together with high concentrations of riboflavin. They suggested that this enzyme served to phosphorylate riboflavin, which in turn served in the deamination of amino acids. Since the enzyme in question is generally regarded as a hydrolyzing, and *not* synthesizing, enzyme, the acceptability of their hypothesis is doubtful. Its possible role in the reabsorption of glucose in the insect Malphigian tubule may be more significant. Bradfield ('46) indicated that a strong alkaline phosphatase histochemically observed in the silk glands of the goat moth, *Cossus cossus,* and in a species of common spider, signified that the enzyme had an important role in the production of fibrous proteins. Day ('49), also from histochemical observations on alkaline phosphatase in several species of insects, suggested that lack of conspicuous enzyme activity in the insect midgut indicated that the role of the enzyme in transporting glucose, etc., across a gradient barrier, attributed to this enzyme in the intestine of mammalian forms, is questionable in insects.

In a highly significant discussion, Moog ('46a) suggested that a high concentration of the acid phosphatase, in the absence of the alkaline enzyme indicates an "adaptation to an active glycolytic mechanism," by a dephosphorylating mechanism operating in the acid range matching this active traffic in glycogen, as is seen in the liver cells of vertebrates. On the other hand, she further states that the common phosphoesterases are *not* involved in the energetic metabolism of carbohydrates, in light of recent studies. In a recent report one of us (Rockstein, '50a) reviewed evidence by Heller ('36), Chadwick and Gilmour ('40) and Williams et al. ('43) which indicated that metabolism, including that of glycogen, is closely related to motor activity and physiological fatigue, especially in flying insects. A further quantitative relation-

ship has been indicated between metabolic activities, like respiration and glucose utilization, and the cholinesterase-acetylcholine system in vertebrates by Welsh and Hyde ('44). The latter system has in turn been quantitatively related to the degree of development of motor activity in vertebrates by Lindeman ('45) and in insects by Rockstein ('50). Thus the high activity of acid phosphatase, compared to the alkaline enzyme, in the honey bee may indicate an adaptive mechanism in the intermediary metabolism of carbohydrates, as yet poorly understood, in insects with highly developed motor mechanism like the strong flyers. These data suggest a fertile field for further study on the metabolism of carbohydrates in the adult honey bee, including the occurrence and transformation of intermediates reported in other insect species, as well as the more precise role of ATP.

SUMMARY

1. The activity of an enzyme system, present in total homogenate of the adult worker honey bee and hydrolyzing sodium β-glycerophosphate was studied under different experimental conditions.

2. A pronounced maximum activity was found to occur at pH 5.6, and a less conspicuous secondary maximum at pH 8.4. Acid enzyme activity was considerably greater than the alkaline.

3. Activation by $MgCl_2$ was observed in both acid and alkaline ranges.

4. The alkaline enzyme showed maximum activity at 35°C., under the conditions of study.

5. In both acid and alkaline ranges, hydrolysis of the glycerophosphate substrate occurred at a rate twice as great as that for disodium phenyl phosphate hydrolysis.

6. The possible role of this enzyme system in the intermediary carbohydrate metabolism of insects with well-developed motor mechanisms like strong flight is discussed briefly.

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COMMENTS AND COMMUNICATIONS

Comments relating to articles which have recently appeared in the Journal of Cellular and Comparative Physiology and brief descriptions of important observations will be published promptly in this Section. Preliminary announcements of material which will be presented later in more extensive form are not desired. Communications should not in general exceed 700 words.

HISTOCHEMISTRY OF MUSCLE CHOLINESTERASE AFTER MOTOR NERVE SECTION [1]

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ELEVEN FIGURES

Manometric studies of cholinesterase (ChE) activity in skeletal muscle after motor nerve section have led to conflicting data. Reports of decreased concentration (Couteaux and Nachmansohn, '38; Stoerk and Morpeth, '44) and increased concentration (Marnay and Nachmansohn, '37) and a decreased followed by an increased concentration (Sawyer et al., '50) after nerve section have appeared. This paper re-

[1] This work was supported in part by a research grant from the National Institutes of Health, Public Health Service.

[2] Henry Strong Denison Scholar, 1950–51.

ports the results of histochemical studies of muscle ChE after motor nerve section.

Ten albino rats of 200–250 gm were anaesthetized with ether, the brachial plexus on the left side exposed and the ulnar and median nerves resected in the upper arm for a distance of 2 cm. At intervals of 2 to 39 days after operation the rats were sacrificed, the flexor muscles below the elbow removed and treated histochemically as reported elsewhere (Koelle and Friedenwald, '49; Kupfer and Koelle, '51). Con-

TABLE 1

Changes in size of ChE-staining area of motor end-plates and of muscle fibers after motor nerve section [1]

DAYS AFTER MOTOR NERVE SECTION	CHE-STAINING AREA OF MOTOR END PLATE				MUSCLE FIBER		
	Length	Width	Area		Width	Cross-Sectional Area	
			mm²	Percent Control		mm²	Percent Control
	μ	μ			μ		
Control	52 ± 3 [2]	31 ± 2	1.6 ± 0.2	..	53 ± 3	2.2 ± 0.3	.
9	50 ± 2	25 ± 1	1.3 ± 0.1	23	41 ± 3	1.3 ± 0.2	39
16	36 ± 2	14 ± 1	0.5 ± 0.1	69	33 ± 1	0.9 ± 0.1	61
39	29 ± 2	16 ± 2	0.5 ± 0.1	72	25 ± 2	0.5 ± 0.1	77

[1] The values presented represent the average of ten micrometer readings.

[2] $SE = \sqrt{\frac{\Sigma d^2}{N(N-1)}}$

trol studies were done on the unoperated side. The motor end plate configuration staining for ChE activity and the muscle fibers were measured by use of a micrometer. (See table 1.)

The end plate area staining for ChE activity decreased about 23% in the first 9 days (figs. 1, 3), about 69% by the 16th day (fig. 5) and about 72% by the 39th day after motor nerve section (fig. 10) as compared with the end plate area from the unoperated side (fig. 9). It is not possible to estimate ChE *activity* quantitatively from these data.

The muscle cross-sectional area decreased about 39% in the first 9 days (figs. 2, 4), about 61% by the 16th day (fig. 6) and about 77% by the 39th day (fig. 11).

It would seem that the overall change in ChE concentration and total ChE content of skeletal muscle after motor nerve section can either increase, decrease or remain constant. This would depend upon the rate of change of ChE per unit dry weight which is concentrated within the volume of the motor end plate on the one hand, and the rate of change of the muscle volume on the other. Such determinations are not obtainable by this method at present.

SUMMARY

Histochemical study of muscle ChE revealed that the motor end plate configuration associated with ChE activity decreased about three-fourths in area by the 39th day after motor nerve section, the greater portion of the decrease occurring within the first 16 days.

I am greatly indebted to Dr. H. B. van Dyke for the facilities of his laboratory and interest in the work and to Dr. George B. Koelle for the encouragement and helpful suggestions during the course of this investigation. I wish to express my gratitude to Mr. Delbert Parker for the photomicrographs.

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PLATE 1

EXPLANATION OF FIGURES

All photomicrographs are longitudinal sections of rat skeletal muscle stained for cholinesterase activity and do not necessarily represent the average area for each time period after motor nerve section. Counterstained preparations are indicated as such. All sections are 25 μ thick.

1 Two days after motor nerve section, × 600. End plate encircles tip of arrow.

2 Same as in figure 1, × 125. Counterstained with hematoxylin-eosin.

3 Nine days after motor nerve section, × 600.

4 Same as in figure 3, × 125. Counterstained with hematoxylin-eosin.

5 Sixteen days after motor nerve section, × 600. There is a diminution in the area of the end plate configuration as compared with figures 1 and 3.

6 Same as in figure 5, × 125. Counterstained with hematoxylin-eosin. A decrease in size of the end plate configuration as compared to figures 2 and 4 is noted.

7 Twenty-two days after motor nerve section, × 600.

8 Same as in figure 7, × 125. Counterstained with hematoxylin-eosin.

9–11 Muscle taken from the same rat. The muscle in figure 9 is from the control side and is normal with complete motor innervation. The muscle in figures 10 and 11 are from the denervated side. Compare figures 9 and 10 as to the area of the motor end plate configuration.

9 The motor nerve which is intact, × 600.

10 Thirty-nine days after motor nerve section, × 600.

11 Same as in figure 10, × 125. Counterstained with hematoxylin-eosin.


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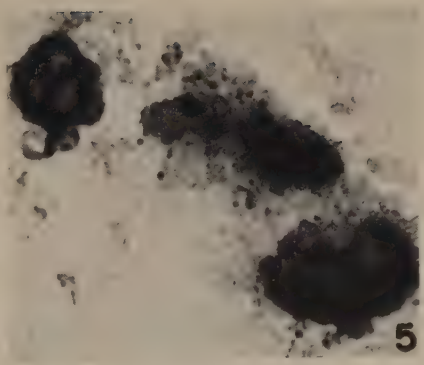
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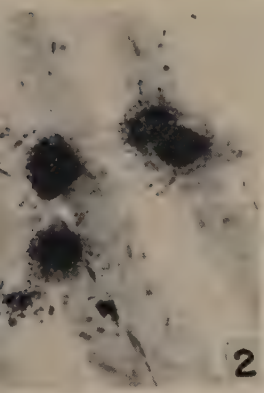
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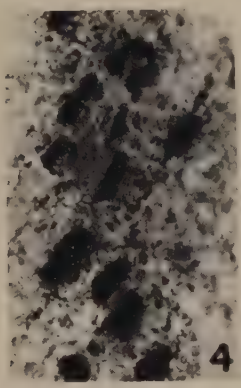
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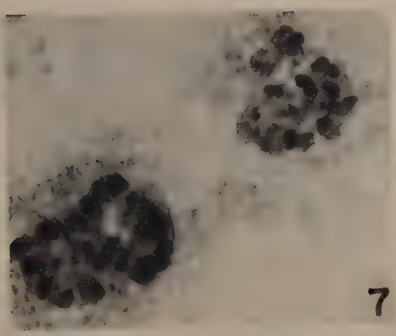
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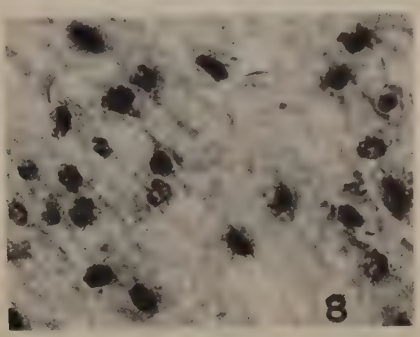
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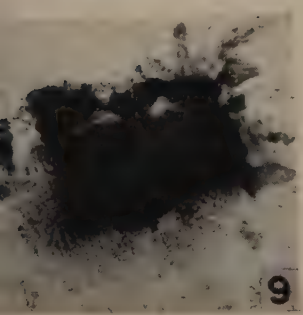
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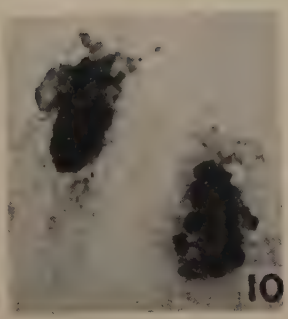
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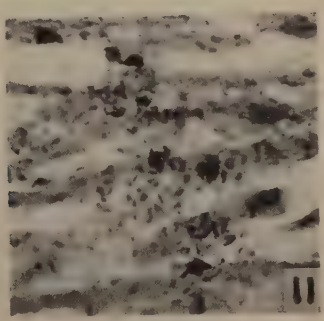
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NOTICE TO CONTRIBUTORS

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It is recognized that prompt publication is essential, and the aim will be to issue papers within three months of acceptance.

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